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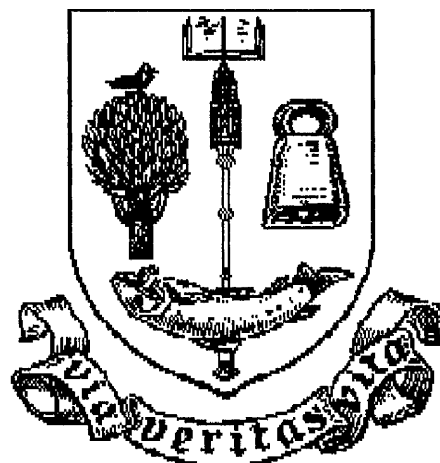
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Analysis of virulence related determinants of *Aeromonas salmonicida*
using transposon mutagenesis.

Mourad Djebara

Presented for the Degree of Doctor of Philosophy in the Faculty of Science,
University of Glasgow

Department of Microbiology
March 1994

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Thesis
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To my parents and my brothers and sisters.

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Index of figures

Figure 1 : Diagram of the cell wall of *A. salmonicida*.

- " 2 : Interrelationship between haemolytic and caseinase activities of *A. salmonicida*.
- " 3 : Mechanisms of lysis of trout erythrocytes by T-lysin.
- " 4 : Structure and general organization of Tn5.
- " 5 : The restriction map of the A-protein gene of *A. salmonicida* .
- " 6 : The restriction map of the serine protease gene of *A. salmonicida* .
- " 7 : Viability of *A. salmonicida* 80628 and *E. coli* HB 101 in BHI broth supplemented with furazolidone.
- " 8 : Agarose gel electrophoresis of *A. salmonicida* Tn5 mutants.
- " 9 : Protease assay of *A. salmonicida* Tn5 mutants.
- " 10 : SDS-PAGE analysis of fresh culture supernatants of *A. salmonicida* Tn5 mutants.
- " 11 : Characteristics of growth of *A. salmonicida* TnphoA mutants.
- " 12 : Growth of *A. salmonicida* 4700 and mutant Pa3 on Coomassie Blue agar plates.
- " 13 : Protease assay of *A. salmonicida* TnphoA mutants (Shaken cultures).
- " 14 : " " " " " " (Static cultures).

Figure 15 : Haemolytic activity of *A. salmonicida* 4700 TnphoA mutants on horse blood agar plates.

- " 16 : Haemolytic activity of *A. salmonicida* 4700 TnphoA mutants on salmon red blood cells.
- " 17 : Effects of EDTA and CaCl₂ on the activity of *A. salmonicida* glycerophospholipid:cholesterol acyltransferase and *Clostridium perfringens* phospholipase C.
- " 18 : GCAT activity of *A. salmonicida* TnphoA mutants.
- " 19 : SDS-PAGE analysis of fresh culture supernatants of *A. salmonicida* TnphoA mutants.
- " 20 : SDS-PAGE analysis of TnphoA mutants (culture supernatants).
- " 21 : " " " " " " "
- " 22 : SDS-PAGE analysis of whole cells of TnphoA mutants.
- " 23 : SDS-PAGE analysis of outer membrane proteins of TnphoA mutants.
- " 24 : Gelatin-PAGE of fresh culture supernatants of TnphoA mutants.
- " 25 : SDS-PAGE analysis of The A-protein digest of TnphoA mutants.
- " 26 : Immunoblot analysis of culture supernatants of *A. salmonicida* TnphoA mutants.
- " 27 : " " " " " " " "
- " 28 : Immunodiffusion test of fresh culture supernatants of TnphoA mutants.

- " 29 : Agarose gel electrophoresis of restriction digest of plasmid pGLW50.
- " 30 : Southern blot analysis of *Ecor* I digested chromosomal DNA of *A. salmonicida* TnphoA mutants.
- " 31 : Agarose gel electrophoresis of *A. salmonicida* TnphoA mutants.
- " 32 : Electron micrographs of *A. salmonicida* TnphoA mutants.
- " 33 : Electron micrographs of *A. salmonicida* TnphoA mutants.
- " 34 : SDS-PAGE analysis of LPS of *A. salmonicida* TnphoA mutants.

Index of tables

Table 1 : Taxonomic position of the principal bacterial pathogens of teleost fish.

- " 2 : List of drugs and compounds for the control of bacterial fish diseases.
- " 3 : Differential characteristics of the Vibrionaceae family genera.
- " 4 : Differentiation between *A. salmonicida* species.
- " 5 : Differentiating characteristics of *A. salmonicida* strain used in this study.
- " 6 : Characteristics of *E. coli* strains used in this study.
- " 7 : Characteristics of transposon delivery vectors.
- " 8 : Antibiotic resistance markers of *E. coli* HB 101 and *A. salmonicida* 80628.
- " 9 : Frequencies of formation of *A. salmonicida* 80628 Km^R colonies.
- " 10 : Continued from table 9.
- " 11 : Frequencies of spontaneous mutations of *A. salmonicida* to kanamycin.
- " 12 : Frequencies of expression of transposons Tn5 and Tn7 in *A. salmonicida* 80628.
- " 13 : Sensitivity of *A. salmonicida* TnphoA mutants to kanamycin.
- " 14 : Frequencies of TnphoA mutagenesis of *A. salmonicida* 4700.
- " 15 : Haemolytic titres of fresh culture supernatant of TnphoA mutants against horse blood.
- " 16 : Haemolytic titres of fresh culture supernatant of TnphoA mutants against salmon red blood cells.
- " 17 : Differentiating characteristics of *A. salmonicida* 4700 TnphoA mutants.

List of abbreviations

³² P	: Radioisotope phosphorus.
<i>abcA</i>	: Gene encoding for a 34 kD protein which is believed to belong to the ABC transport family of protein.
ASH1	: Gene encoding for a haemolysin.
ASH3	: Gene encoding for a haemolysin.
BCIP	: 5-bromo-4-chloro-3-indoyl phosphate.
BHI	: Brain-heart infusion.
BHISMA	: Brain-heart infusion-skimmed milk agar.
BSA	: Bovine serum albumin.
cfu	: Colony forming unit.
CMP-KDO	: CTP:CM P -3-deoxy-manno-octulosonate cytidylyltransferase.
DNA	: Deoxyribonucleic acid.
DPC	: Dioctanoylphosphatidylcholine.
ECP	: Extracellular products.
EDTA	: Ethylenediamine tetra-acetic acid.
GCAT	: Glycerophospholipid:cholesterol acyltransferase.
kb	: Kilobase.
kD	: Kilodalton.
Km	: Kanamycin.
LCAT	: Lecithin:cholesterol acyltransferase.
LCL	: Leucocytolytic factor.
LPS	: Lipopolysaccharides.
mA	: Milliamps.
nm	: Nanometer.

OD	: Optical density.
OMP	: Outer membrane protein.
PBS	: Phosphate buffered saline.
PMSF	: Phenylmethanesulphonylfluoride.
RBC	: Red blood cell.
SDS	: Sodium dodecyl sulphate.
SDS-PAGE	: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis.
<i>vapA</i>	: The A-layer gene.
μ l	: Microlitre.
$^{\circ}$ C	: Degree celsius.

Index of contents.

Acknowledgments	i
Index of figures	ii
Index of tables	iii
Abbreviations	iv
Summary	v

Introduction.

	Page
General introduction	1
1 - Furunculosis.	5
1.1 - Discovery and history.	5
1.2 - Pathological features of furunculosis.	6
1.3 - The causative agent of furunculosis.	7
1.3.1 - History and classification of the organism.	7
1.3.2 - Virulence related factors of <i>A. salmonicida</i> .	11
1.3.2.1 - Cell wall associated virulence factors.	11
1.3.2.1.1 - The A-layer.	11
1.3.2.1.2 - Endotoxins of <i>A. salmonicida</i> .	14
1.3.2.1.3 - Outer membrane proteins and siderophores.	14
1.3.2.2 - Proteases.	15
1.3.2.3 - Glycerophospholipid:cholesterol acyltransferase (GCAT).	16
1.3.2.4 - Haemolysins.	18
1.3.2.5 - Salmolysin.	21
1.3.2.6 - Leukocytolytic factor (LCL).	21

1.4 - Interrelationship between virulence factors of <i>A. salmonicida</i> .	22
1.5 - Control of furunculosis by Vaccines, immunogenicity of <i>A. salmonicida</i> virulence factors.	22
2 - Transposon mutagenesis.	28
2.1 - Translocable genetic elements.	28
2.2 - Transposon mutagenesis in bacteria.	29
2.3 - Genetics of <i>A. salmonicida</i> .	33
2.4 - Cloning of <i>A. salmonicida</i> genes.	34

Materials & Methods

3.1 - Bacterial strains.	39
3.1.1 - <i>A. salmonicida</i> strains.	39
3.1.2 - <i>E. coli</i> bacterial strains.	39
3.2 - Growth conditions and media.	39
3.3 - Maintenance of bacterial cultures.	39
3.4 - Bacteriological methods.	42
3.4.1 - Filter conjugation.	42
3.4.2 -Antibiotic-resistance screening.	44
3.5 -Measurement of bacterial growth.	44
3.6 - Protein estimation.	44
3.7 - Autoagglutination test.	44
3.8 - Phage sensitivity test of <i>A. salmonicida</i> TnphoA mutants.	45
3.9 - Preparation of agar plates.	45
3.9.1 - Congo red agar plates.	45
3.9.2 - Egg-yolk agar plates.	45
3.9.3 - Coomassie brilliant blue agar plates.	46
3.9.4 - Preparation of "sandwich" plates.	46
3.9.5 - Horse blood agar plates.	46
3.9.6 - Selection plates.	46

3.10 - Biochemical Assays.	47
3.10.1 - Caseinase assay.	47
3.10.2 - Conductimetric enzyme assay of the phospholipase activity of the GCAT.	47
3.11 - Haemolytic titration.	48
3.12 - SDS-polyacrylamide gel electrophoresis of proteins.	48
3.12.1 - Preparation of samples for SDS-PAGE.	49
3.12.2 - Preparation of samples for gelatin-PAGE.	49
3.12.3 - Electrophoresis of protein samples.	49
3.13 - Analysis of gels after electrophoresis.	50
3.13.1 - Coomassie blue staining.	50
3.13.2 - Silver staining of proteins.	50
3.13.3 - Silver staining of LPS.	51
3.13.4 - Staining of gelatin gels.	51
3.14 - Partial peptide mapping of the A-protein.	51
3.15 - Immunological techniques.	52
3.15.1 -Western blotting.	52
3.15.2 - Ouchterlony gel diffusion test.	53
3.16 - DNA analysis techniques.	53
3.16.1 - Agarose gel electrophoresis of DNA.	53
3.16.2 - Plasmid DNA preparations.	54
3.16.2.1 - Kado and Liu procedure.	54
3.16.2.2 - Plasmid midi preparation.	54
3.16.3 - Miniprep of bacterial genomic DNA.	54
3.16.4 - Southern blotting and hybridization.	55
3.16.5 - Hybridization conditions.	56
3.16.6 - Restriction endonuclease digestion.	56
3.16.7 - Preparation of restriction fragment length probe.	56
3.16.8 - Random hexanucleotide labelling of probes.	57

3.16.9 - Estimation of DNA molecular size.	57
3.17 - LPS extraction.	57
3.18 - Extraction of outer-membrane proteins.	58
3.19 - Electron microscopy of <i>A. salmonicida</i> TnphoA mutants.	58

Results.

4.1- Antibiotic sensitivity of mutants generated using suicide plasmid pUW 964.	60
4.2 - Frequencies of transfer of Tn5 plasmid donors.	62
4.3 - Spontaneous kanamycin-resistance in <i>A. salmonicida</i> 80628.	67
4.4 - Transposon mutagenesis of <i>A. salmonicida</i> 80628 using different vectors.	67
4.5 - Expression of transposon Tn5 and Tn7 in <i>A. salmonicida</i> Tn5 mutants.	67
4.6 - Characteristics of Km-resistant colonies.	70
4.7 - Plasmid analysis of <i>A. salmonicida</i> 80628 Tn5 mutants.	70
4.8 - Caseinase and Gelatinase activities of <i>A. salmonicida</i> 80628 Tn5 mutants.	72
4.9 - Haemolytic activity of Tn5 mutants.	72
4.10 - SDS-PAGE of culture supernatants of <i>A. salmonicida</i> Tn5 mutants.	72
5 - Characterization of TnphoA mutants.	75
5.1 - Isolation of TnphoA mutants.	75
5.2 - Sensitivity of <i>A. salmonicida</i> TnphoA mutants to kanamycin.	77
5.3 - Frequencies of TnphoA and spontaneous mutations of <i>A. salmonicida</i> strain 4700.	77
5.4 - Characteristics of TnphoA mutants colonies on Coomassie Blue and on Congo Red agar plates.	80
5.5- Phage sensitivity of TnphoA mutants.	80

5.6 - Biochemical characteristics.	82
5.6.1 - Caseinase activity of TnphoA mutants.	82
5.6.2 - H-lysin activity of TnphoA mutants.	82
5.6.3 - Titration of T-lysin activities of TnphoA mutants.	82
5.6.4 - Phospholipase activity of TnphoA mutants.	89
5.7 - Protein analysis.	92
5.7.1 - SDS-PAGE analysis of culture supernatants.	92
5.7.2 - SDS-PAGE of whole cells of <i>A. salmonicida</i> TnphoA mutants.	92
5.7.3 - SDS-PAGE analysis of outer membrane proteins of TnphoA mutants.	92
5.7.4 - Detection of gelatinase in culture supernatants of <i>A. salmonicida</i> TnphoA mutants.	97
5.7.5 - Partial peptide mapping of the A-protein exported by mutants.	97
5.7.6 - Immunological analysis of extracellular proteins of TnphoA mutants.	97
5.7.7 - Immuno-diffusion test of concentrated ECP's of <i>A. salmonicida</i> TnphoA mutants.	101
5.8 - Southern blotting of chromosomal DNA of TnphoA mutants.	101
5.9 - Analysis of plasmids of TnphoA mutants.	108
5.10 - Electron microscope analysis of cell surface of TnphoA mutants.	108
5.11 - LPS analysis of TnphoA mutants.	108

Discussion.

6.1 - Transposon mutagenesis of <i>A. salmonicida</i> 80628.	113
6.2- Expression of caseinase, gelatinase and haemolysins in <i>A. salmonicida</i> Tn5 mutants.	115
6.3- Transposon mutagenesis of <i>A. salmonicida</i> 4700 strain.	116
6.4 - Evidence of TnphoA insertions into <i>A. salmonicida</i> chromosomal DNA.	119
6.5 - Alteration of caseinase expression in TnphoA mutants.	121

6.6 - Analysis of other virulence factors of *A. salmonicida*

TnphoA mutants.

123

References.

135

Appendices.

162

SUMMARY

Isolates of *A. salmonicida* were subjected to transposon mutagenesis to analyse virulence determinants produced by the organism. Initially, the work was focused on finding a suitable delivery shuttle for transposon Tn5 and preliminary results suggested that the presence of low molecular weight plasmids in *A. salmonicida* and/or high frequency of excision of Tn5 were the main obstacles to creating Tn5 mutants. Greater success in obtaining mutants was achieved using the suicide plasmid pRT733. Several different classes of mutants were obtained and those which failed to express the serine protease were selected for further studies. The frequency of appearance of these mutants was similar to those reported in the literature for Tn5.

The main objective of this study was to create mutants lacking expression of the 70 kD serine protease, which was reported by many workers to be an important virulence factor in furunculosis in Atlantic salmon. Protease negative mutants were analysed for the expression of other potential virulence determinants such as glycerophospholipid:cholesterol acyltransferase, haemolysins, the A-layer and lipopolysaccharides.

Serine protease-negative mutants created by TnphoA mutagenesis exhibited low activities of the glycerophospholipid:cholesterol acyltransferase and T-lysin, factors which have been considered important in furunculosis; also, expression of the H-lysin was impaired due to the lack of expression of the 70 kD protease by these mutants, thus confirming results reported by several previous workers. Analysis of caseinase-negative mutants indicated that other caseinase (s) might be expressed by the organism, thus, the role of these caseinase (s) in furunculosis should be considered.

Analysis of the organisation of the A-layer in TnphoA mutants indicated that maintenance and assembly of the A-protein (the precursor of the A-layer) was closely linked to the lipopolysaccharide expressed by the organism; loss of a certain type of lipopolysaccharide, shown by loss of sensitivity to the different phages used in this study, resulted in the export of A-protein into culture fluid and disorganization of the A-layer on the surface of the organism; no loss of the A-layer was observed in these mutants.

Results obtained in this study suggested that expression of the 70 kD caseinase and a certain type of lipopolysaccharide were governed by a common regulatory control system; also, *A. salmonicida* may require expression of its serine protease to attain higher levels of activities of other virulence factors which could enable the organism to invade and infect its host.

Introduction

Like all other animals, fish are susceptible to microbial diseases and these are a global problem affecting freshwater and marine, feral, cultured, sport fish, and even ornamental fish. The problem is of major importance when fish are maintained in intensive aquaculture systems.

In the last 20 years, aquaculture, involving a small number of freshwater species of fish, has developed to become a significant source of high quality protein food, which is relatively inexpensive to produce. The world's aquaculture production is estimated to have more than doubled in the past few years and fish represent more than two thirds of this aquaculture output.

For a long time, it has been known that a number of eukaryotic microorganisms, many bacteria, and viruses produce diseases resulting in heavy mortalities in both wild and cultured fish. The bacterial pathogens of major importance are gram-negative; representatives of 25 species have been implicated in fish diseases of which 16 were identified as gram-negative bacteria, described as short rods belonging to the families Enterobacteriaceae, Pseudomonadaceae or Vibrionaceae (Austin & Austin 1987). The present taxonomic positions of the recognized bacterial pathogens of fish are summarized in Table 1.

Furunculosis is a septicaemic infection, principally of salmonids, but *Aeromonas salmonicida*, the causative agent, has also been isolated from non-salmonid fish (Bucke 1979). Experimentally-induced furunculosis showed a range of non-salmonid fish, such as roach (*Rutilus rutilus* L), rudd (*Scardinius erythrophthalmus* L), carp (*Cyprinus carpio* L), goldfish (*Carassius auratus* L) and perch (*Perca fluviatilis* L), can be infected by *A. salmonicida* (Bucke 1980).

The control of diseases in fish farms is of great importance to fish culture; the process requires the maintenance of environmental conditions favourable to the fish, such as adequate levels of dissolved oxygen, correct water temperature, removal of waste,

Table 1 : Taxonomic position of the principal pathogens of teleost fish.

Family	Genus	Species
Cytophagaceae (Gram-negative gliding bacteria)	<i>Flexibacter</i>	<i>F. columnaris</i> <i>F. psychrophila</i> * <i>F. maritimus</i> * unclassified myxobacteria
Enterobacteriaceae (Gram-negative facultatively anaerobic rods)	<i>Edwardsiella</i>	<i>E. ictaluri</i> <i>E. tarda</i>
Pasteurellaceae (Gram-negative facultatively anaerobic rods)	<i>Yersinia</i> <i>Pasteurella</i>	<i>Y. ruckeri</i> <i>P. piscicida</i> *
Pseudomonadaceae (Gram-negative aerobic rods)	<i>Pseudomonas</i>	<i>P. fluorescens</i> <i>P. anguilliseptica</i>
Vibrionaceae (Gram-negative facultatively anaerobic rods)	<i>Aeromonas</i>	<i>A. hydrophila</i> <i>A. salmonicida</i> (including <i>H. piscium</i>)
	<i>Vibrio</i>	<i>V. anguillarum</i> <i>V. ordalli</i> * <i>V. carchariae</i> * <i>V. damsela</i> * <i>V. salmonicida</i> * <i>V. vulnificus</i> biogrp 2
Uncertain (Gram-negative aerobic rods)	<i>Alteromonas</i>	<i>A. piscicida</i>
Bacillaceae (Gram-positive spore-forming bacilli)	<i>Flavobacterium</i> <i>Clostridium</i>	<i>Flavobacterium</i> ssp. <i>C. botulinum</i>
Coryneform group (Gram-positive pleomorphic rods)	<i>Renibacterium</i>	<i>R. salmoninarum</i>
Streptococcaceae (Gram-positive cocci)	<i>Streptococcus</i> Group B <i>Streptococcus</i>	<i>S. faecalis</i> ssp. and haemolytic ungrouped streptococci
Mycobacteriaceae (Acid alcohol fast bacilli)	<i>Mycobacterium</i>	<i>M. chelonae</i> <i>M. fortuitum</i> <i>M. marinum</i>
Nocardiaceae (weakly acid-fast, Gram-positive, branching bacilli)	<i>Nocardia</i>	<i>N. asteroides</i> <i>N. kansasii</i> *
Chlamydiaceae		Epitheliocystis organism (obligatory intracellular parasites)

* : Not described in Bergey's manuals (1974; 1984; 1986).

From Austin and Austin (1987).

avoidance of crowding and disinfection of eggs (Snieszko & Axelrod, 1971). All these husbandary stresses are significant and contribute to susceptibility to disease.

The costs incurred in controlling and preventing diseases of fish in aquaculture systems are very difficult to assess; it is believed that chemotherapy is the only effective means of saving an infected population when all preventive measures have failed .

Since the 1920's a wide range of drugs has been used on fish farms. With the development of aquaculture, the range of antibiotics (table 2) used in fisheries has increased rapidly but many drugs have been banned because they are of little benefit and/or are toxic to fish. Furthermore, the extensive use and abuse of drugs has resulted in an increase in bacterial drug resistance such that a microflora resistant to medically important compounds could possibly develop, which , in turn could transmit the resistance to human pathogens. Among the medically important drugs which should be excluded from fisheries are cycloserine, doxycycline, ethionamide, isoniazid, minocycline, and rifampicin, which are used to control tuberculosis; ampicillin, bacitracin, and kanamycin, which are used in the treatment of staphylococcal infections; chloramphenicol, which is the antibiotic of choice for the treatment of typhoid fever; streptomycin, which is used to cure bubonic plague and gonorrhoeae; furazolidone, a good treatment for intestinal infections, and nitrofurantoin, which is used to treat urinary tract infections. Consideration should, therefore, be given to finding means of discouraging or preventing the use of such drugs in aquaculture (Austin 1980) ; previous studies indicated that the extensive use of sulphonamides resulted in the appearance of drug resistant strains of *A. salmonicida* (Herman 1972). Mainly for these reasons, chemotherapy is restricted to prophylactic use where fish may be at risk from bacterial infection over a short time period or when transferred to a totally new environment.

Table 2: List of drugs and compounds for the control of bacterial fish diseases; medically important drugs are deleted from the table (Adapted from Austin 1985).

Bacterial diseases	Antimicrobial compounds
Acinetobacter disease	Iodophors.
Bacterial kidney disease	Erythromycin, iodophors, penicillin G, sulfisoxazole, sulphamethazine, clindamycin hydrochloride, spiramycin.
Carp erythrodermatitis	Furazolidone, oxytetracycline.
Cold water disease	Diquat, furanace, oxytetracycline, quaternary ammonium compounds, sulfisoxazole, sulphamerazine, sulphamethazine.
Columnaris	Acriflavin-neutral, diquat, furanace, malachite green, oxolinic acid, oxytetracycline, quaternary ammonium compounds, sulfisoxazole, sulphamerazine, sulphamethazine.
Edwardsiellosis/emphysemateous putrefactive disease of catfish	Oxytetracycline.
Fin rot	Albucid, aquarol, benzalkonium chloride, copper sulphate, furanace, globucid, malachite green, oxytetracycline, phenoxethol.
Flavobacteriosis	Iodophors.
Furunculosis	Flumequine, fosfomicina, iodophors, nifurprazine, oxolinic acid, oxytetracycline, potentiated sulphonamides, sulfisaxole, sulphamerazine, sulphamethazine.
Gill disease	Benzalkonium chloride, diquat, furanace, malachite green, oxytetracycline, quaternary ammonium compounds, sodium chloride.
Haemorrhagic septicaemia	Furanace, iodophors, oxolinic acid, oxytetracycline, sulphamerazine.
Mycobacteriosis	Chloramine B or T, sulfisoxazole.
Nocardiosis	Sulphonamides.
Salmonid blood spot	Methylene blue/oxytetracycline.
Streptococcosis	Oxytetracycline, sodium nifurstyrenate.
Ulcer disease	Oxolinic acid, oxytetracycline.
Vibriosis	5,7 -dichloro-8-hydroxyquinoline, halquinol, furanace, nifurprazine, oxolinic acid, oxytetracycline, sulfisoxazole, sulphamerazine, sulphamethazine.

From B. Austin (1985).

1 - Furunculosis

1.1 - Discovery and history

Furunculosis is the most important bacterial disease in most salmonid farming countries. It was first described from a fish farm in Germany in 1894 by Emmerich & Weibel, thereafter there was a series of outbreaks of the disease in France, Austria, Belgium, Switzerland, Great Britain and Ireland (McCarthy & Roberts, 1980). The disease was first reported in the U.K. in 1911, and it was the subject of an official inquiry by the British government in the 1920's and the 1930's, because of the severe losses caused in the Scottish east coast rivers. The three official reports presented by Mackie *et al.* (1930, 1933, 1935) provided the basis of much of our present knowledge (McCarthy, 1977), and the disease is now recognized as worldwide in distribution.

Some devastating epizootics of furunculosis have been recorded in wild fish populations but the major economic impact of the disease in recent years has been on salmonid cultivation (Hastings, 1988). The disease has been, and remains, of great importance to those involved in salmon culture, its effects on other species in the natural environment are not well documented. Active epizootics of furunculosis have been reported during winter (Klontz & Wood, 1972), and outbreaks more frequently occur during the summer (Mackie *et al.*, 1930, 1933, 1935). Some environmental factors, such as oxygen tension, water temperature, and water salinity, are important in increasing the susceptibility of fish to microbial pathogens, and water milieu can also facilitate the transmission of these pathogens.

It is generally accepted that the disease occurs by lateral transmission of the bacterium *A. salmonicida* (McCarthy, 1977) which is also associated with two bacterial septicaemia syndromes in non-salmonids, the post-traumatic septicaemia in centrarchids and the carp erythrodermatitis syndrome; the transmission of disease via contaminated water was demonstrated by McCarthy (1977). However, infected fish or fish farm materials are all possible sources of infection as carcasses of fish that have succumbed to furunculosis are heavily contaminated with *A. salmonicida*; consequently, the presence of

such fish constitutes a significant source of contamination. Also, healthy carrier fishes have long been suspected of being involved in the transmission of furunculosis (McCarthy & Roberts, 1980). Detection of clinical disease in these asymptomatic carriers is usually achieved by combination of corticosteroid injections and cultivation of the fish at elevated temperature (Bullock & Stuckey, 1975); using this technique, Scallan and Smith (1985) found that more than 60 % of fish carried *A. salmonicida* without signs of the disease. Also McCarthy and Roberts (1980) reported that brown trout from different commercial fish farms had a carrier rate in the range of 40 - 80 %. It is likely that the mud and detritus present in earth-bottomed fish ponds or even in rivers will become contaminated with *A. salmonicida* following an epizootic of furunculosis (McCarthy, 1977).

Attempts to demonstrate vertical transmission of furunculosis have failed, however, McCarthy (1977) described a positive isolation of *A. salmonicida* from the interior of fertile eggs from naturally-infected mature fish. He also demonstrated the possibility of carrier fish transmitting the bacterium with their sex products.

1.2 - Pathological features of furunculosis

Several disease forms of furunculosis have been described and McCarthy & Roberts (1980) described the development of the disease in four forms : peracute, acute , subacute and chronic.

In the peracute form, death of the fish can be very rapid, and this condition is usually restricted to fingerling fish, especially young *Salmo salar* , in hatcheries fed with water containing carrier wild fish. Losses in farmed fish may be very high, and McCarthy & Roberts (1980) concluded that death in most cases is probably due to cardiac damage.

The acute form of the disease is characterized either by severe toxic septicaemic changes with rapid mortality and the development of furuncles or merely the standard features of an acute bacterial septicaemia, i.e. darkening in color, inappetance, lethargy, small haemorrhages, and skin lesions ; in the last two stages of the disease (subacute &

chronic) the lesions are generally similar and are common in older fish which are generally lethargic and have one, or more, obvious furuncles on the flank or dorsum.

1.3 - The causative agent of furunculosis

1.3.1 - History and classification of the organism

A. salmonicida is a gram-negative, facultatively anaerobic, non-motile organism. The cell size was estimated to be in the range of 1.3 - 2 by 0.8 - 1.3 μm and in the latest edition of Bergey's Manual of Determinative Bacteriology (1984) it is classified in the family Vibrionaceae, which consists of four distinct genera (Table 3): *Vibrio*, *Aeromonas*, *Photobacterium*, and *Pleisiomonas*. In the early literature, *A. salmonicida* was recognized as *Bacillus salmonicida*; many workers, such as Griffin *et al.* (1953), suggested that *B. salmonicida* should be classified in the genus *Aeromonas*, a suggestion subsequently implemented by Snieszko in the 7th edition of Bergey's Manual of Determinative Bacteriology (Breed 1957).

Smith (1963) suggested that *A. salmonicida* should be removed from the genus *Aeromonas* to a newly-created genus, *Necromonas*; she also suggested the creation of a new species, *Necromonas achromogenes*, after she found non-pigmented strains of *A. salmonicida*. On the basis of insufficient variance between pigmented and non-pigmented *A. salmonicida* isolates, Schubert (1967) disagreed with her proposal to create a new species and reclassified the organism as *A. salmonicida*, subspecies *achromogenes*. Kimura (1969) isolated a non-pigmented strain which was considered to differ from existing *A. salmonicida* strains; he proposed the name *A. salmonicida* subspecies *masoucida*.

The classification of atypical strains of *A. salmonicida* was the subject of many reports, e.g. Evelyn (1971), Schubert (1974) and McCarthy (1975); these authors described atypical strains which shared many characteristics with *A. salmonicida*. Using various numerical taxonomy computer analyses, McCarthy proposed a classification of these three organisms in three groups based on the host-range of fish infected, the first

group consisted of typical *A. salmonicida* strains, the second group contained atypical strains isolated from salmonid fish, while the last group was composed of a new group of atypical strains which are associated with disease syndromes of non-salmonid fish (McCarthy & Roberts, 1980). Popoff (1984) classified organisms included in the genus *Aeromonas* into two well-separated groups, the first consisting of psychrophilic and non-motile aeromonads with the second group including mesophilic and motile aeromonads; as cited above, *A. salmonicida* strains are differentiated into three subspecies : *A. salmonicida* subspecies *salmonicida*, *A. salmonicida* subspecies *achromogenes* and *A. salmonicida* subspecies *masoucida*. These strains can be distinguished by biochemical tests such as the production of brown water-soluble pigment, indole production, aesculin hydrolysis and utilisation of L-arabinose (Table 4), biochemical studies showed that all strains produced acid from glucose and maltose but not from xylose, dulcitol, inositol, adonitol, malonate and mucate. They also possess gelatinase, deoxyribonuclease, ribonuclease, and Tween 80 esterase, but do not produce hydrogen sulphide from thiosulfate (Popoff 1984).

Motile *Aeromonas* strains (*A. hydrophila*, *A. caviae*, *A. sobria*) are rod-shaped occurring singles or in pairs. These bacteria exhibit monotrichous flagellation, and growth on nutrient broth occurs at 37 °C. However, *A. salmonicida* strains are not motile and appear as coccobacilli in pairs, chains and clumps; they grow at 37 °C with different changes such as inhibition of pigment production at temperatures above 30 °C; in addition, the ability to produce catalase and to degrade aesculin, DNA, elastin and gelatin is lost at 37 °C (McIntosh & Austin, 1991). Popoff (1984) reported that optimum growth temperature of *A. salmonicida* is between 22 °C and 25 °C and the organism usually ferments arabinose, trehalose, galactose, mannose and dextrin, and its growth is inhibited in KCN broth and nutrient broth containing 7.5 % NaCl. Strains of *A. salmonicida* from uncomplicated cases of furunculosis can be isolated and identified on trypticase soy agar and on selective media for Enterobacteriaceae. No specific selective medium has been developed for the isolation of *A. salmonicida* and it is usually recognisable from the

Table 3: Differential characteristics of the Vibrionaceae family genera.

(Baumann & Schubert, 1984).

Symbols : +, all positive; [+], most positive; -, all negative; D, variable.

Characteristics	Vibrio	Photobacterium	Aeromonas	Pleisiomonas
Sheathed polar flagella .	+	-	-	-
Accumulation of poly- β -hydroxybutyrate coupled with the inability to utilize β -hydroxybutyrate.	-	+		
Na+ is required for growth or stimulates growth.	+	+	-	-
Production of Lipase	[+]	D	+	-
Utilization of D-Mannitol	[+]	-	[+]	-
Mol % G+C of DNA	38 - 51	40 - 44	57 - 63	51

Table 4: Differentiation between *A. salmonicida* species (Popoff, 1984).

Symbols : +, typically positive; -, typical negative; d, differs among strains.

A. salmonicida subsp.

Characteristics	<i>salmonicida</i>	<i>achromogenes</i>	<i>masoucida</i>
Motility	-	-	-
Monotrichous flagellation in liquid medium.	-	-	-
Lophotrichous flagellation in liquid medium.	-	-	-
Cocci in pairs, chains and clumps.	+	+	+
Rods in singles and pairs.	-	-	-
Brown water-soluble pigment.	+	-	-
Growth in nutrient broth at 37°C.	-	-	-
Indole production in 1 % peptone water.	-	+	+
Esculin hydrolysis.	+	-	+
Growth in KCN broth (Møller technique).	-	-	-
L-Histidine and L-Arginine utilization.	-	-	-
L-Arabinose utilization.	+	-	+
Fermentation of salicin.	d	d	d
Fermentation of sucrose.	-	+	+
Fermentation of mannitol.	+	-	+
Breakdown of inositol.	-	-	-
Acetoin from glucose (Voges-Proskauer).	-	-	+
Gas from glucose.	+	-	+
H ₂ S from cysteine.	-	-	+

development of small colonies producing oxidase and brown pigmentation. It has been found that *A. salmonicida* species are serologically homogenous and freshly isolated cells autoagglutinate when suspended in saline. However, if maintained in culture for a long period of time in the laboratory or cultured at elevated temperatures, many strains lose their autoagglutinating properties, and also their virulence (Hastings 1988) due to the loss of the A-layer (see later).

1.3.2 - Virulence related factors of *A. salmonicida*

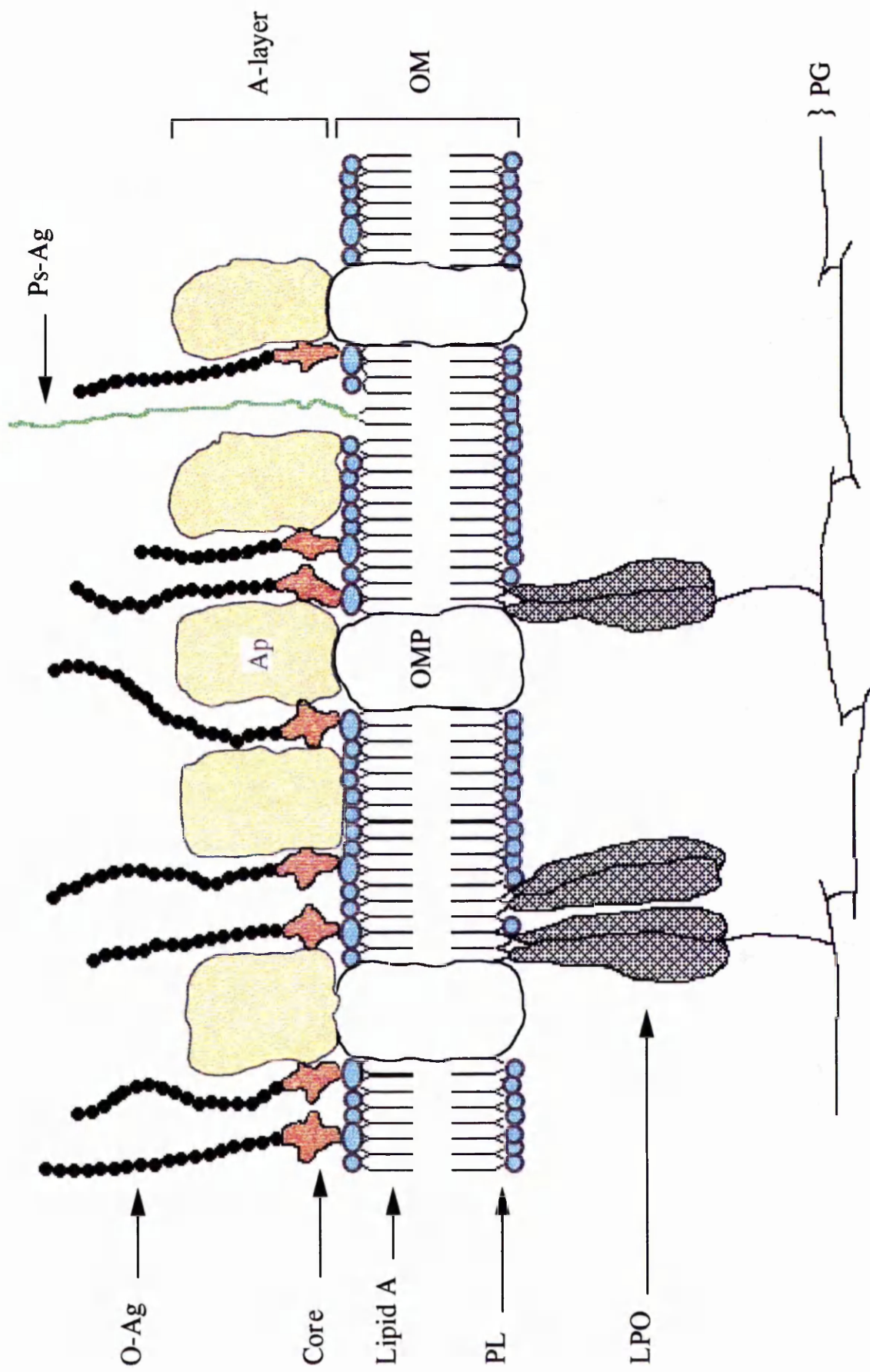
Several putative virulence factors such as A-layer, proteases, leucocytotoxins, GCAT and haemolysins have been demonstrated which may be responsible for the pathological features of the disease in fish. Extracellular products (ECP) of *A. salmonicida* possessing haemolytic, proteolytic and leucocytolytic activity were lethal to rainbow trout when injected intraperitoneally (Munro *et al.*, 1980). Destruction of the leucocytes of blood and leukopenia by the production of leucotoxins was reported by Evenberg *et al.* (1986) and Fuller *et al.* (1977). Ellis *et al.* (1981) reported the role of ECP, and especially the role of protease and leucocytolytic activity, in reproducing the pathological features observed in fish affected with furunculosis, but few of these virulence factors are well characterized and found to be directly implicated in pathogenicity because of the complexity of the ECP; it should be noted that studying the virulence factors is of great importance in the understanding of the pathogenicity of *A. salmonicida*, also, the study of the relationship between the bacterium and its host may be the most interesting step in the comprehension of the virulence mechanisms of *A. salmonicida*.

1.3.2.1 - Cell wall associated virulence factors

1.3.2.1.1 - The A-layer

The cell surface protein array designated the A-layer is a major cell envelope antigen of *A. salmonicida* and was first described by Udey & Fryer (1978). The A-layer consists of a protein of a molecular weight of 49 kD, termed the A-protein (Trust *et al.*,

Figure 1: Diagram of the cell wall of *A. salmonicida* , based on the model of Evenberg *et al.* (1985). The outer membrane (OM) and the A-layer are external to the peptidoglycan (PG) of the cell wall. The A-layer consists of regularly arranged subunits of the A-protein (Ap). Lipopolysaccharide (LPS) is composed of an O-antigen (O-Ag), core and a lipid A moiety which is inserted in the outer membrane. An additional polysaccharide antigen (Ps-Ag) may also be inserted in the outer membrane. LPO : lipoprotein; PL: phospholipid. (reproduced by kind permission of authors's publisher).



1980a; Kay *et al.*, 1981; Evenberg & Lugtenberg, 1982) which constitutes a refractile protein barrier essential for virulence (Phipps *et al.*, 1983); it also acts as a barrier to the release of ECP such as protease and haemolysins (Titball & Munn, 1985). The A-layer was found to be arranged as a tetragonal paracrystalline array on the surface of *A. salmonicida* (Kay *et al.*, 1981; Udey & Fryer, 1978) which appears to be crucial for the ability of typical strains of *A. salmonicida* to cause furunculosis, because isogenic mutants generated by growth at elevated temperature, which are unable to produce A-layer, displayed a significant reduction in virulence (Ishiguro *et al.*, 1981; Adams *et al.*, 1988). Subsequently, the A-layer was shown to be responsible for causing autoagglutination of *A. salmonicida* cells (Ishiguro *et al.*, 1981), which is apparently due to the hydrophobicity conferred by the A-layer (Trust *et al.*, 1983)

The layer appears to be multifunctional; it protects *A. salmonicida* from the bactericidal activity of both immune and non-immune serum (Munn *et al.*, 1982), and from bacteriophage (Ishiguro *et al.*, 1981), it displays binding capabilities with macrophages (Trust *et al.*, 1983), immunoglobulins (Phipps & Kay, 1988), porphyrins (Kay *et al.*, 1985), congo red (Ishiguro *et al.*, 1985), laminin and fibronectin (Doig *et al.*, 1992), as well as collagen type IV (Trust *et al.*, 1993). The A-layer was reported to be structurally conserved, and each cell contains approximately 5×10^5 molecules of A-protein (Kay *et al.*, 1984).

A recent study (Stewart *et al.*, 1986) using computer image analyses of electron micrographs of *A. salmonicida* A-layer, demonstrated the presence of two conformations of the layer in two-dimensional mass distribution projections; also a three-dimensional image reconstruction analysis has shown that the subunits are comprised of two morphological domains, a heavy mass domain with a linker arm to a domain of lesser mass (Dooley *et al.*, 1989). Belland and Trust (1987) and Chu *et al.* (1991) succeeded in cloning and sequencing the structural gene for the A-protein in *E. coli*.

1.3.2.1.2 - Endotoxins of *A. salmonicida*

Lipopolysaccharides (L.P.S) or endotoxins of *A. salmonicida* are also recognized as virulence factors; the L.P.S of *A. salmonicida* is composed of three moieties : lipid A, a core oligosaccharide and an O-polysaccharide (O-antigen) (figure 1). The major role of L.P.S in the virulence of *A. salmonicida* is resistance to the host's normal serum bactericidal mechanisms and this is mediated by the O-antigen polysaccharide component of the molecule. Both low and high molecular weight L.P.S were detected in the *A. salmonicida* cell surface layer by Munn *et al.* (1982); some of the O-polysaccharide chains penetrate the A-layer and are exposed at the cell surface (Chart *et al.*, 1984). It is also suggested that these chains serve as a framework around which the A-layer array is assembled (Dooley *et al.*, 1985); these L.P.S were found to be remarkably homogeneous when compared to similar smooth L.P.S extracted from *E. coli* and *S. typhimurium* (Chart *et al.*, 1984).

A polysaccharide-like antigen found on the surface cell of *A. salmonicida* and termed Ps-antigen (see figure 1) was reported by Evenberg *et al.* (1985); also a possible formation of a capsule in *A. salmonicida* was observed when the bacterium was cultivated in liquid medium with excess of glucose as source of carbon (Garrote *et al.*, 1992). This structure could be similar to that described by Evenberg *et al.* (1985) but the role of exopolysaccharidic structures or Ps-antigen is unclear though it is believed that it may mediate adherence of the bacteria to the host tissues.

1.3.2.1.3 - Outer membrane proteins and siderophores

Studies on outer membrane proteins of *A. salmonicida* and their role in the pathogenesis of furunculosis are relatively few. However, reports have indicated that *A. salmonicida* produced blebs or vesicles which contained outer membrane proteins (MacIntyre *et al.*, 1980); these vesicles may be involved in secretion and represent a means of packaging enzymes such as toxic lipid components which play a role in pathogenesis. Thus, GCAT association with outer membrane proteins was reported by MacIntyre and

Buckley (1978). Analysis of outer membrane proteins of *A. salmonicida* strains from different sources did not reveal differences when compared to *A. hydrophila* outer membrane profiles (Aoki & Holland, 1985) which showed a great diversity. Leblanc *et al.* (1981) reported that outer membrane proteins of *A. salmonicida* were restricted to a single serogroup, whereas the serotyping of *A. hydrophila* was polymorphous. Also the properties of outer membrane proteins have been studied and some of them with a molecular weight ranging from 35 - 45 kD displayed characteristics similar to *E. coli* porins (Aoki & Holland, 1985; Darveau *et al.*, 1983).

An additional defence mechanism involving the binding of free iron to siderophores was reported by Hirst *et al.* (1991); such a system could create iron-restricted conditions within the host (Martinez *et al.*, 1990). *A. salmonicida* strains grown under conditions of iron-restriction produced two types of iron uptake systems, siderophore-mediated mechanisms and siderophore-independent mechanisms; interestingly, typical strains of *A. salmonicida* were found to use the siderophore mediated system and atypical strains used the siderophore-independent mechanisms in sequestration of iron. The siderophore produced by typical strains was partially purified and behaved as a 2,3-diphenolate-cathecol with differences in chromatographic properties and chemical nature (Hirst *et al.*, 1991). In addition, the A-protein has been implicated as being a component of an iron-uptake mechanism, and the binding site for the porphyrins, haemin and protoporphyrins IX could be responsible for these mechanisms (Kay *et al.*, 1985).

1.3.2.2 - Proteases

It has been reported in the early literature that members of the family Vibrionaceae produce extracellular proteases (Dahle 1969; 1971). Among the extracellular proteins (E.C.P) produced by *A. salmonicida*, proteases were considered to be significant virulence factors of the bacterium. Shieh & Maclean (1975) reported a proteolytic enzyme in the culture supernatant of *A. salmonicida* and after isolation and purification of the

enzyme, Mellergaard (1983) studied its physical properties and sensitivity to different inhibitors; activity was inhibited by phenylmethylsulphonylfluoride (PMSF).

Sheeran & Smith (1981) found that *A. salmonicida* produces at least two separate extracellular proteolytic activities, a serine protease which is inhibited by PMSF, and probably a metallo-enzyme which is inhibited by ethylenediaminetetra-acetic acid (EDTA). These findings were supported by Hastings and Ellis (1985) who called the serine protease P1 and the metallo-enzyme P2; they also detected caseinase and gelatinase activities which were possibly due to a single serine protease which is dependent on divalent cations for its activity. Rockey *et al.* (1988) succeeded in isolating and purifying the two activities indicating that *A. salmonicida* produces more than two gelatinases. As P1 can digest both casein and gelatin their conclusions support the results of Sheeran and Smith (1981).

The serine protease has an estimated M.W. of 70 kD and was considered to be one of the most important virulence factors in furunculosis by Sakai (1977); this conclusion was later supported by Cipriano *et al.* (1981), Ellis (1981), and Shieh (1982). The role of the 70 kD serine protease was studied by several workers (Fyfe, 1988; Sakai, 1985; Rockey *et al.*, 1988), and it seems that most workers agree that the 70 kD serine protease is responsible for tissue destruction and lesion formation in salmonids (Sakai, 1985; Fyfe *et al.*, 1986). Also, it may play an important role in supplying *A. salmonicida* cells with amino acids and thus stimulate growth (Sakai, 1985). The protease is resistant to most of the protease inhibitors present in normal fish serum, though it is inhibited by α 2-macroglobulin (Ellis, 1987). Recently Whitby *et al.*, (1992) cloned and sequenced the structural gene of the 70 kD protease in *E. coli*.

1.3.2.3 - Glycerophospholipid:cholesterol acyltransferase (GCAT)

All members of the family Vibrionaceae have been found to produce and export the enzyme glycerophospholipid:cholesterol acyltransferase (GCAT) except the species *Pleisiomonas shigelloides* (MacIntyre *et al.*, 1979). The GCAT could be considered a

candidate to replace some of the lipases currently used in biotechnological applications (Harwood, 1989).

The purified enzyme exhibits three enzymatic activities and it shares a number of characteristic features with mammalian lecithin:cholesterol acyltransferase (LCAT); the three activities, phospholipase, acyltransferase, and lysophospholipase were inseparable during purification. The enzyme catalyzes the transfer of the sn-2 fatty acid of lecithin to cholesterol (MacIntyre & Buckley, 1978), and can catalyze acyl transfer from a variety of other donors, including *p*-nitrophenyl esters (Buckley, 1983; Bonelli & Jonas, 1989).

The bacterial phospholipases characterized to date exhibit one position specificity, but the GCAT exhibited two position specificities, both as an acyltransferase and as a phospholipase; also, no divalent cation is required for the activity of the enzyme (Buckley, 1982), but it is activated by albumin and Apolipoprotein-A-I (Apo-A-I); albumin is thought to activate by complexing with lysolecithin and fatty acid (Fielding *et al.*, 1972), whereas Apo-A-I appears to interact selectively with the surface of the liposome substrates (Chung *et al.*, 1979). It seems that the GCAT is less specific than cobra venom phospholipase-A₂ (Roberts *et al.*, 1978) and mammalian LCAT (Glomset & Nommi, 1973); the enzyme has a strong preference for phospholipids carrying short-chain or unsaturated fatty acids as acyl donors, and the enzyme catalyzes acyl transfer by using other alcohols in addition to cholesterol (Buckley, 1982).

The mechanism of action of the GCAT indicates that acyl transfer depends on the formation of a complex between the acyl donor and the acyl acceptor. Hilton *et al.* (1990) showed that the enzyme is monomeric before proteolytic treatment but it may dimerize after proteolysis. Lee & Ellis (1990) reported that two forms of the GCAT were present in culture supernatants of *A. salmonicida*, with molecular weight of 27 kD and 54 kD. They also showed that GCAT activity is enhanced when the enzyme is associated with lipopolysaccharide (LPS).

The GCAT produced by *A. hydrophila* is a high molecular weight complex with more than one enzyme activity (MacIntyre & Buckley, 1978). The enzyme was larger in size than that of *A. salmonicida* (Hilton *et al.* 1990).

1.3.2.4 - Haemolysins

A. salmonicida was shown to produce two separate haemolytic activities by Titball and Munn (1981). The H-lysin has haemolytic activity against erythrocytes from a wide variety of species with maximum activity against horse erythrocytes, and the T-lysin is lytic to salmonid (trout) erythrocytes only; Hastings and Ellis (1985) confirmed these findings. The M.W. of the T-lysin was estimated to be 56 kD, and the H-lysin was synthesized as an inactive 42.3 kD precursor, which, on proteolytic cleavage, yielded an active 29.5 kD hemolysin (Fyfe *et al.*, 1987). Both enzymes are produced by *A. salmonicida* during the logarithmic phase of growth and are activated and released during the stationary phase. The role of hemolysins in the pathogenicity of *A. salmonicida* is still controversial; Titball and Munn (1983, 1985) reported that T-lysin required the P1 caseinase (70 kD) to cause complete lysis of trout erythrocytes (Figure 2) but Rockey *et al.* (1988) found that complete lysis of erythrocytes from different salmonids did not specifically require the 70 kD caseinase of *A. salmonicida*.

Recently Hirono and Aoki (1991), using DNA-DNA hybridization analysis, found that *A. salmonicida* carries ^{the} same gene encoding for H-lysin of *A. hydrophila*. Very recently, Hirono and Aoki (1993) ^e succeeded in cloning three different haemolysin genes of *A. salmonicida*; these authors reported that two of the three genes were also found in *A. hydrophila* and *A. sobria* and one haemolysin contained some homologous sequence regions of the *Vibrio vulnificus* and *Vibrio cholerae* cytotoxin-haemolysin.

Figure 2: Interrelationship between hemolytic activities of *A. salmonicida* (Titball & Munn, 1985). The pro-H-lysin (42.3 kD) is converted to active H-lysin (29.5 kD) by proteolytic cleavage by the caseinase; the relationship between GCAT and caseinase is unclear. (reproduced by kind permission of the publisher).

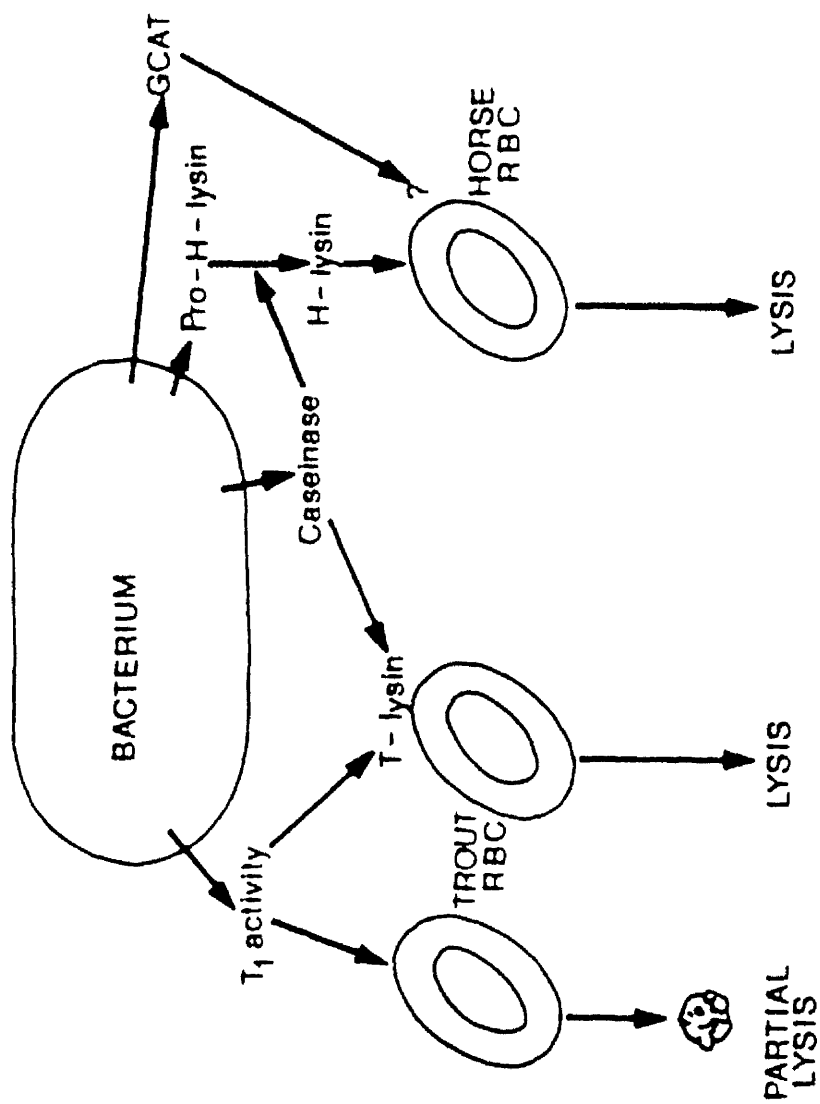
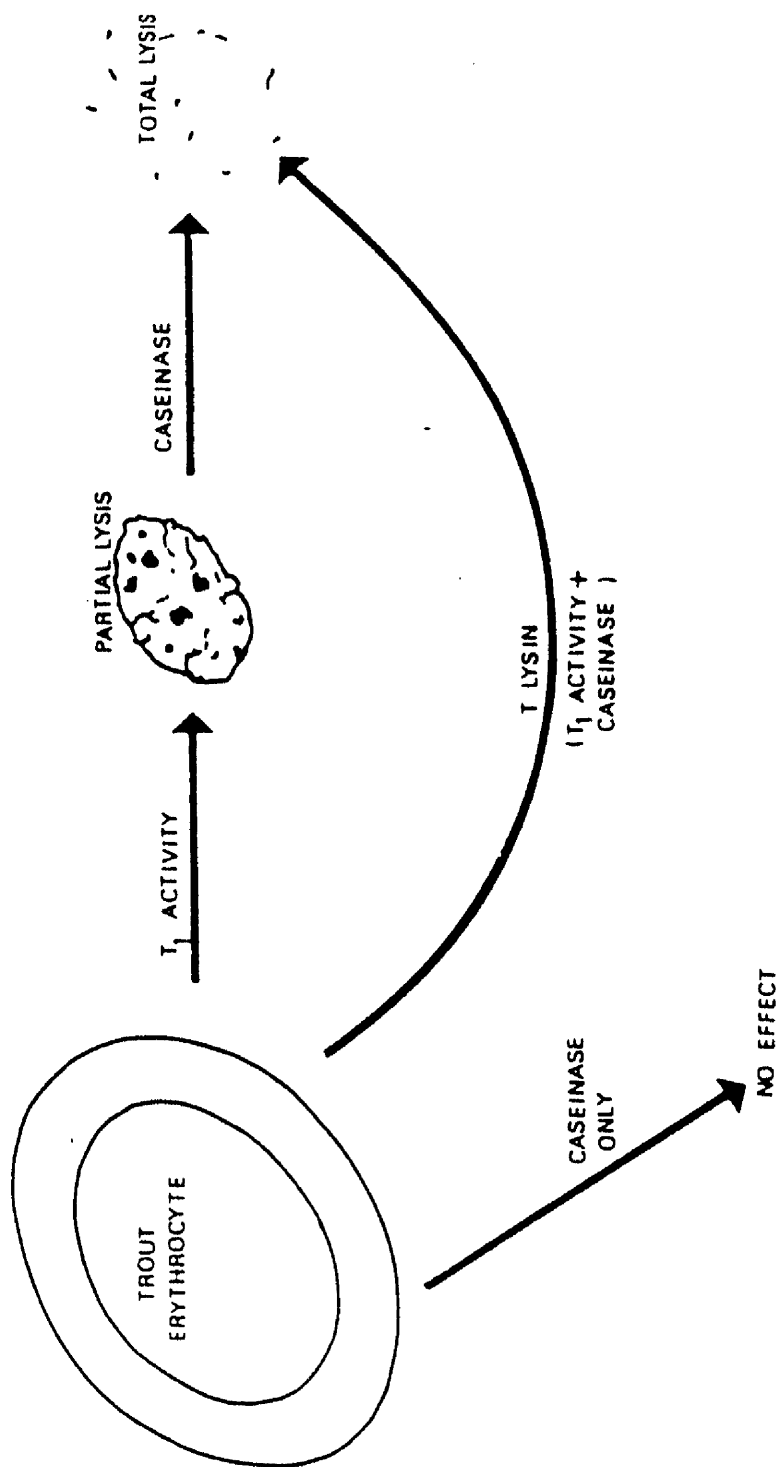


Figure 3: Mechanisms of lysis of trout erythrocytes by T-lysin (Titball & Munn, 1985). The association of caseinase and T1-activity produced a complete lysis of erythrocytes (T-lysin), the absence of caseinase resulted in partial lysis of erythrocytes. (reproduced by kind permission of the publisher).



1.3.2.5 - Salmolysin

A heat-labile protein of about 200 kD which was purified and characterized by Nomura *et al.* (1988) was called salmolysin as it caused complete lysis of salmonid erythrocytes. The enzyme complex contained 68 % carbohydrate and Nomura *et al.* (1988) suggested it was a glycoprotein; it was stable at neutral pH and was inhibited by ferrous ion and reducing agents such as L-cysteine and reduced glutathione. No ionic detergents inactivated the enzyme but it was activated by anionic detergent, such as low concentrations of sodium deoxycholate; subtilisin and trypsin inactivated the enzyme.

1.3.2.6 - Leukocytolytic factor (LCL)

Fuller *et al.* (1977) reported that both virulent and avirulent strains of *A. salmonicida* produced a leukocytolytic factor (LCL) which consisted of one major protein component associated closely with carbohydrates. The M.W. of the LCL factor was 100-300 kD; both virulent and avirulent strains produced identical LCL but the activity of the LCL produced by virulent strains was 10 times greater than that of the avirulent preparations. Injection of purified LCL factor into fish did not cause any fish deaths, but injection of the LCL factor with a low dose of *A. salmonicida* cells resulted in a significant increase in mortalities of fish when compared to that produced by the organism alone (Fuller *et al.*, 1977).

Serological studies showed that LCL factor reacted with monospecific anti LCL-serum and with antiserum against whole cells of *A. salmonicida*, but it did not cross-react with antisera against endotoxin of the organism. Thus LCL factor and endotoxin of *A. salmonicida* appear to be separate and distinct (Fuller *et al.*, 1977).

The LCL factor is regarded as a possible virulence factor, which presumably could increase the susceptibility of the host by preventing effective destruction of bacteria by leucocytes eventually resulting in leucopenia. This may allow spread of bacteria to initiate colonization of other organs of infected fish via the circulation (Ellis *et al.*, 1981).

1.4 - Interrelationship between virulence factors of *A. salmonicida*

Interaction between virulence factors of *A. salmonicida* has been noted above suggesting that the bacterium may exert its toxic effects *in vivo* through a number of factors acting synergistically. Interrelationship studies have mainly concentrated on proteolytic and hemolytic activities. For example, Titball & Munn (1983) reported that complete lysis of trout erythrocytes (T-lysin) was due to the cooperative effect of a heterogenous T1 factor and a caseinase (see figure 3) , this conclusion was supported by other workers (Hastings & Ellis 1985; Titball & Munn 1985; Rockey *et al.*, 1988). It is not clear whether membrane proteins are exposed to the caseinase after treatment with T1 activity or whether this treatment facilitates entry of the caseinase into the cell.

Also, it appears that H-lysin is synthesised as an inactive pro-H-lysin with a M.W. of 42.3 kD which is converted to active H-lysin (29.5 kD) by proteolytic cleavage (Titball *et al.* , 1985).

GCAT complexed with LPS (GCAT/LPS complex) possesses an incomplete hemolytic activity against trout erythrocytes when compared to complete solubilization of erythrocytes membranes by the extracellular products (E.C.P); this is similar to the T1 activity described by Titball & Munn (1985), which acts synergistically with the extracellular protease (caseinase) to produce complete lysis. The association of GCAT with LPS resulted in enhanced hemolytic and lethal activities and heat protection also, since heating the purified toxin resulted in loss of these activities but prolonged incubation of the heated toxin with erythrocytes resulted in the appearance of hemolysis.

1.5 - Control of furunculosis by vaccines and immunogenicity of *A. salmonicida* virulence factors

The control of furunculosis in aquaculture has proven to be complex (Ghittino, 1984). As with other infectious diseases there are four main requirements : management techniques, selective breeding for resistance, chemotherapy and vaccination (Ellis, 1987).

Controlling diseases in fish farms requires adequate planning of the facility as well as attention to husbandry of the fish, water quality control and routine sampling; prevention of furunculosis is extremely important, and prophylaxis requires the exclusion of any possible source of infection starting by disinfecting eggs and elimination of asymptomatic carriers. Detection of *A. salmonicida* in these carriers is difficult; the carrier status in susceptible fish has been examined by McCarthy (1977) and Munro (1982), and a method to eliminate the asymptomatic carrier state of *A. salmonicida* has been developed by Markwardt and Klontz (1989). The epidemiological interest in the carrier state of the organism in fish has also been evaluated by Michel and Faivre (1991); these authors also reported that ectoparasites could play a critical part in natural infection by initiating skin lesion in fish. The use of strains of fish which have been selected for disease resistance could minimize losses, as found for certain strains of rainbow trout which are particularly resistant to furunculosis (Cipriano, 1983).

The therapy used in the treatment of furunculosis is similar to that for many other systemic infectious diseases of fish; drugs such as flumequine, iodophors, oxolinic acid, oxytetracycline, sulfasaxsole, sulfamerazine and sulfamethazine are commonly used to treat furunculosis (Austin, 1985).

The first attempt to immunize fish against furunculosis was reported by Duff (1942) who administered a vaccine consisting of chloroform-killed cells of *A. salmonicida*. Some protection was obtained but subsequently many empirical attempts to immunize fish using vaccines consisting of killed cells of *A. salmonicida* were reported with disappointing results. In the last 20 years research has been concentrated on understanding the biology of the microorganism, its relationship with the fish, and the virulence mechanisms and antigenic composition of the bacterium.

In the last 30 years vaccine trials were carried out by many workers using different types of vaccines and different routes of administration of vaccines; Spence *et al.* (1965), Udey and Fryer (1978), Michel (1979), Palmer and Smith (1980), and Smith *et al.* (1980) used oral administration and parenteral injections of whole killed cells of *A. salmonicida* as

vaccines, which provided inconsistent results. However Duff (1942) and Smith *et al.* (1980) have both shown protection against furunculosis. Immunization of fish using formalin-killed cells of either virulent or avirulent strains of *A. salmonicida* resulted in comparable agglutinins titres (Michel, 1979; Cipriano, 1982; Olivier *et al.*, 1985). Olivier *et al.* (1985) reported that virulent strains of *A. salmonicida* were more immunogenic than avirulent strains used in their studies.

Shieh (1982) indicated that good protection of Atlantic salmon was obtained using crude extracellular products (ECP) and partially purified protease. Ellis *et al.* (1988) reported poor antibodies production in rainbow trout to crude ECP, formalinized ECP, and to ECP inactivated by normal serum, including protease and haemolysin. Also, Hastings and Ellis (1988) reported that no circulating antibodies to haemolysin and protease were found in rainbow trout immunized with either native ECP or formalin-inactivated ECP.

Antigenicity of fractionated ECP and purified antigens was investigated by Udey and Fryer (1978), Paterson (1981), McCarthy *et al.* (1983), Shieh (1985), Hastings and Ellis (1988), and Kawahara *et al.* (1990). Sakai (1985) reported protection of sockeye salmon with partially-purified protease neutralized by normal serum; the same findings were reported by Shieh (1985), who indicated that injection of extracellular protease protected Atlantic salmon from a virulent challenge; in rainbow trout poor antibody production was observed to crude ECP, formalinized ECP, and ECP (including protease and haemolysin) inactivated by normal serum (Ellis *et al.*, 1988). Also administration of bacterins derived from avirulent strain of *A. salmonicida* provided protection to brook trout and Atlantic salmon, but better protection was observed when fishes were injected with bacterins from a virulent *A. salmonicida* culture (Paterson 1981). Paradoxically, it was reported that a fraction of ECP from an avirulent strain was more immunogenic than an equivalent fraction from a virulent strain when administered with Freund's adjuvant (Cipriano 1980).

Many workers have indicated that the presence of serum agglutinins was not correlated with immunity (Michel, 1979; Cipriano, 1982, 1983; Olivier *et al.*, 1985).

Using native ECP as the immunogen, Kawahara *et al.* (1990) failed to detect antibodies against protease but found that haemolysin reacted with antibodies produced in rabbits; the same authors indicated that salmolysin was significantly immunogenic in fish, while protease was very weakly, or not, immunogenic.

Purified lipopolysaccharides (LPS) from *A. salmonicida* were shown to be immunogenic and induced production of specific antibodies (Paterson & Fryer 1974; Chart *et al.*, 1984; Rockey *et al.*, 1991). Evenberg *et al.* (1985) reported that both high and low molecular weight LPS of *A. salmonicida* were immunogenic when injected into rabbits; another constituent of the cell wall of *A. salmonicida*, the A-protein, also induced antibodies production (Munn *et al.*, 1982; Evenberg *et al.*, 1985).

Generally there has been no correlation between protection and a specific immune response, and there was no agreement among authors to suggest the use of particular protocols. In the last few years the routes of administration of vaccines have developed, such that oral administration, intraperitoneal and intramuscular injections, hyperosmotic infiltration and bath immersion are commonly used. Recently, fractions extracted from *A. salmonicida* have been used in vaccine trials; these fractions include ECP of which the antigenic composition was not defined, whole cells plus ECP, which might be expected to contain a maximum number of different antigens, live attenuated vaccines (Vaughan *et al.* 1993), which are basically non-autoagglutinating and avirulent strains of *A. salmonicida*, glycoprotein vaccines, protease vaccines and endotoxin vaccines which consist of purified antigens, and commercial vaccines (Hastings 1988). In the majority of laboratory trials, vaccinated fish have been challenged with the same bacterial isolate used in vaccine preparation, and it remains to be shown that protection would be equivalent against other isolates (Hastings 1988). However, none of these vaccines have been reported to induce specific long-term immunity against furunculosis; in the meantime, the development of an ideal furunculosis vaccine remains a challenging objective.

Freshly isolated virulent strains of *A. salmonicida* from acute furunculosis may possess a minimum lethal dose as low as 10^2 - 10^3 viable cells (McCarthy 1975). Trust

(1986) reported that intramuscular injection of viable cells possessing the A-layer resulted in death of all fish used in their experiments within 3-4 days, whereas fish injected with as many as 10^6 cells lacking the A-layer did not kill fish. This indicates the role of A-layer in protecting *A. salmonicida* from the fish's nonspecific defenses.

Cell-mediated immunity has been reported when fish are injected with *A. salmonicida*; Smith *et al.* (1980) showed that lymphocytes from fish immunized against *A. salmonicida* produced the lymphokine macrophage migration inhibitory factor. Teleost fishes produce antibodies which are restricted to one class of immunoglobulin, resembling mammalian IgM, and can activate the classical complement pathway for cytolysis (Ellis, 1982). For many years the occurrence and persistence of agglutinating antibodies was considered to indicate protection against furunculosis (Spence *et al.*, 1965). However researchers who had observed protection after immunization of fish emphasized that the presence of humoral antibodies was not necessary for protection.

Intraperitoneal or intramuscular injections and hyperosmotic infiltration of whole killed or disrupted cells of *A. salmonicida* have generated conflicting results (Spence *et al.*, 1965; Palmer & Smith, 1974; Udey & Fryer, 1978; Michel, 1979; Smith *et al.*, 1980; Palmer & Smith, 1980; Newman & Majnarich, 1985). Discrepancies between the observations of different authors may be partially explained by variables in experimental conditions such as fish species, temperature of water during immunization, and during the challenge injections, and preparation procedure of the bacterins (Michel, 1982). It is believed that antibodies produced by fish when injected with whole killed or disrupted cells, are induced principally by the A-layer (A-protein), since this is the most abundant external antigen exposed to the fish immune system.

Several workers have reported that injection of fish with crude active ECP or purified components of ECP (protease, LPS, or glycoprotein) could confer some protection of fish against furunculosis in laboratory trials.

The endotoxin of *A. salmonicida* is strongly antigenic, and an immune response could be observed in fish inoculated with LPS. It seems that agglutinating antibodies

synthesized by fish during conventional immunization are induced by the antigenic determinants of the endotoxin (Paterson & Fryer, 1974). The endotoxin of *A. salmonicida* cell wall demonstrates similar toxic properties to the endotoxins of other Gram-negative bacteria (Paterson & Fryer, 1974; Pol *et al.*, 1981) and the stimulating antigen was the LPS of the cell wall (Pol *et al.*, 1981). Although the endotoxin from various *A. salmonicida* cultures seems to contain the same main antigenic determinants, this antigen does not appear to constitute the whole immunogen. Also, McCarthy & Roberts (1980) reported that LPS does not play a major role in the pathogenesis of furunculosis. Recently, Rockey *et al.* (1991) using monoclonal antibodies against *A. salmonicida* LPS, demonstrated that antigenic variation exists among different isolates. The antigenic determinants of *A. salmonicida* LPS were located on the O-polysaccharide, which cross-reacted with both anti-LPS monoclonal and polyclonal antibodies (Chart *et al.*, 1984; Dooley *et al.*, 1985); similarities between antigenic determinants of *A. salmonicida* and *A. hydrophila* LPS were found by Dooley *et al.* (1985) and polyclonal antibodies prepared to LPS of *A. hydrophila* displayed antigenic cross-reactivity with epitopes of the O-polysaccharide of *A. salmonicida*.

The immunogenicity of *A. salmonicida* extracellular protease was demonstrated by Shieh (1985) who found that injection of partially purified extracellular protease of *A. salmonicida* protected Atlantic salmon from a virulent challenge; in contrast Ellis *et al.*, (1988) reported that antibody protection in rainbow trout to E.C.P containing protease was extremely poor.

2 - Transposon mutagenesis

2.1 - Translocable genetic elements

Transposable elements or transposons are DNA segments able to promote their own movement to new genetic locations by processes which require neither extensive DNA sequence homology between the element and the site of insertion nor the *rec* genes needed for the classical homologous crossing-over (Berg & Berg, 1983); many such elements have been found in prokaryotes and eukaryotes and their size ranges from 1 kb (IS1) to over 48 kb (bacteriophage lambda).

The simplest are the insertion sequence (IS) elements, which generally consist only of the genes and sites needed for the transposition. Much more complex are the transposing bacteriophages, such as lambda and Mu, that carry genes needed for phage growth and lysogenization as well as integration (Berg *et al.*, 1989). The nomenclature, physical and genetic properties, ecology and mechanisms of translocation of transposable elements have been reviewed by several workers (Starlinger, 1980; Kleckner, 1981; Shapiro, 1983; Berg & Berg, 1983).

Transposable elements are generally characterized by terminal repeat DNA sequences (either direct or inverted) flanking genes that encode enzymes which promote transposition (transposase). The transposition of these elements is independent of host cell recombination systems. They have many properties, and the following characteristics are common to most, if not all, transposable elements (Foster & Kleckner 1980):

i)-Transposons are capable of serial translocations. Thus, transposons can move from one replicon to another serially without changing their structure.

ii)-The replicons participating in translocation need not have any nucleotide sequences in common.

iii)-Translocation occurs at normal frequencies in hosts deficient in homologous recombination.

iv)-Transposons have characteristic structures. Most have been shown to possess inverted repeat sequences at their termini which can form loop structures in electron microscopy heteroduplex experiments. Some elements have long inverted repeat sequences (e.g Tn5 & Tn10) while others have short inverted sequences (e.g TnA & IS1).

v)-Transposons can insert at many sites in the recipient replicon. Most show some degree of clustering in preferred regions, at preferred sites or both.

2.2 - Transposon mutagenesis in bacteria

Mutagenesis with transposons provides a better approach than chemical mutagenesis to obtaining mutants for different studies such as virulence, analysis of metabolism, analysis of developmental genes, etc. It is also a powerful technique for investigating the structure and function of bacterial genes (Duggleby *et al.*, 1990). The earliest examples of transposon mutagenesis were carried out on *Escherichia coli* and *Salmonella typhimurium* (Kleckner *et al.*, 1977).

Transposon mutagenesis can be divided into two broad categories (Duggleby *et al.*, 1990):

- i)-Random transposon mutagenesis.
- ii)-Site-directed mutagenesis.

In random transposon mutagenesis, a transposon is introduced into a recipient cell via a suitable delivery system, allowing the construction of a population of mutant strains with random transposon insertions throughout the genome. The phenotype of the mutants can then be analyzed and classified, and the desired ones are selected for further studies. This class of mutagenesis is normally used for initially locating on the genome, genes, or gene clusters, of interest and for the preliminary analyses of their organization.

The second category involves cloning the region of DNA under study (which can be from either prokaryotes or eukaryotes) into a multi-copy plasmid in *E. coli*. The cloned DNA is then subjected to high density transposon mutagenesis, before being re-introduced back in to the original host. There, the mutagenised sequences are selected for integration

into the chromosome via homologous recombination, to replace the natural (mutagenised) DNA, this allows the effect of the mutagenised DNA on the phenotype to be studied.

The main limitation of transposon mutagenesis is that insertions into essential genes cannot be isolated. Transposon-induced mutations are extremely stable; reversion by precise excision of the inserted transposon occurs at very low frequencies and can easily be recognized since it is accompanied by the loss of the transposon marker (Simon, 1989). Any mutagenesis experiment requires an appropriate transposon delivery vector which must transfer to the recipient at frequencies high enough to produce transconjugants and must not be maintained in the recipient (Duggieby *et al.*, 1990). Also, there must be an efficient route by which the vector carrying the transposon can be introduced into the recipient bacterium; a phage vector is optimal since it can be injected into every cell of a culture but the conjugative cell transfer of suitable plasmids is the most feasible way to introduce a transposon into the recipient (Simon, 1989).

The first transposable elements to be used for genetic analysis in bacteria were the *E. coli* bacteriophages, Lambda and Mu (Campbell, 1971). The discovery of antibiotic resistance transposons led to an explosion in the use of transposable elements for *in vivo* genetic engineering in diverse bacterial species. These elements carried resistance markers that were much easier to select than Mu and Lambda (Berg *et al.*, 1989). Both gram-negative and gram-positive bacteria were investigated using transposon mutagenesis showing a wide spread use of transposable elements in many bacterial species. Several workers, e.g. Mills (1985), have reviewed the potential use of transposon mutagenesis in plant pathogenic gram-negative bacteria, also Simon (1989) reviewed transposon mutagenesis in non-enteric gram-negative bacteria. It should be noted that among the transposons used, transposon Tn5 received more attention and gained the widest acceptance because of the advantages it offers when compared to other transposons (Berg & Berg, 1983).

Tn5 was first detected in bacteriophage lambda grown in a strain of *E. coli* carrying an R-factor derived from a clinical isolate (Berg *et al.*, 1975). It encodes an

aminoglycoside-3'-phosphotransferase II (Jorgensen *et al.*, 1979), which confers resistance to the commonly used antibiotics neomycin (Neo) and kanamycin (Km); in addition there is a gene coding for resistance to metalloglycopeptides antibiotics like bleomycin (Ble) (Mazodier *et al.*, 1985), which is followed by a streptomycin phosphotransferase gene that is expressed to various degrees in several non-*E. coli* strains (O'Neill *et al.*, 1984). Thus, if selection for resistance to neomycin or kanamycin is not sufficient, the presence of Tn5 can be verified unambiguously by using one of the other resistance markers instead of, or in addition to, kanamycin and neomycin.

The molecular weight of Tn5 was estimated to be 5.8 kb and it contains two inverted repeat sequences of 1,533 pairs base (bp) each (figure 4 A); generally, insertion of Tn5 into bacterial genomes is stable, but excision occurs at low frequencies (10^{-8} to 10^{-4}) (Berg, 1989), and transposition occurs at high frequencies (10^{-2} to 10^{-5}) (Berg & Berg, 1983). Also, the IS elements in Tn5 are independently mobile (Berg *et al.*, 1982).

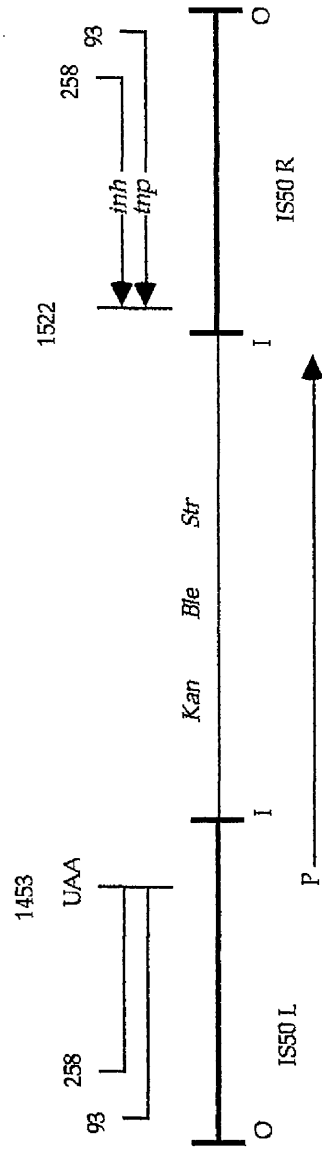
A variety of Tn5 derivatives have been constructed, which combine the advantageous transposition properties of Tn5 with other useful features; Tn5-Tc (Simon *et al.*, 1983) contains in addition to kanamycin and neomycin the tetracycline resistance gene. Similarly, Tn-Ap (Ampicillin) and Tn5-Cm (Chloramphenicol) were constructed by Sasakawa & Yoshikawa (1987). Also, Tn5-lac carries the *lac* operon lacking the transcriptional start signal (Kroos *et al.*, 1984) and TnphoA (see figure 4 B) which contains an alkaline phosphatase gene without its signal peptide allowing identification by insertional mutagenesis of genes whose products are secreted (Manoil & Beckwith, 1985).

A detailed restriction endonuclease cleavage map of Tn5 has been generated (Jorgensen *et al.*, 1979), and the DNA sequence of the IS50 plus about half of Tn5 central region, including the kanamycin gene, is known (Beck *et al.*, 1982).

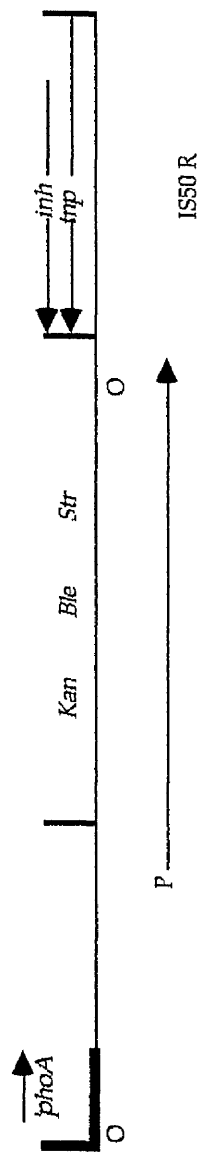
Figure 4: A)-Structure and general organization of Tn5. The 1,533-bp insertion sequences IS50 left (L) and IS50 right (R) are present as terminal inverted repeats in Tn5; O, outside end of IS50; I, inside end of IS50; P, promoter for expression of central resistance genes; kan, kanamycin resistance; ble, bleomycin resistance; str, streptomycin resistance (which is not expressed in *E.coli*); 93 and 258, the start sites for translation of transposase and inhibitor proteins, respectively, in nucleotides from the O end; 1453 UAA, the ocre codon in IS50 L; 1522, the stop site for translation of transposase (*tnp*) and inhibitor proteins (*inh*) (Berg *et al.* 1989).

B)- TnphoA is a derivative of Tn5 with a region encoding *E.coli* alkaline phosphatase minus the signal sequence and expression signals, inserted into the left IS50 element. Insertion of TnphoA generates hybrid proteins composed of alkaline phosphatase fused to amino-terminal sequences of other proteins (Manoil & Beckwith 1985).

A): Tn5



B): TnphoA



2.3 - Genetics of *A. salmonicida*

DNA homology studies of *A. salmonicida* strains revealed that at the species level, *A. salmonicida* contains two groups, the so-called "typical" strains from an extremely homogeneous genetic group with a sequence homology level ranging from > 87% to 100% homology (Belland & Trust, 1988; McInness *et al.*, 1979); the "atypical" isolates formed a more diverse group than the "typical" isolates with sequence homology ranging from 60-90%. DNA base composition of the two groups ranged from 55.4 +/- 1.6 to 55.7 +/- 1.8 mol % G-C (Belland & Trust, 1988).

DNA probes for *A. salmonicida* were used by Hiney *et al.* (1990) to facilitate the study of the ecology of the organism and the etiology of furunculosis; these studies suggested that the DNA sequences of *A. salmonicida* are strongly conserved (Hennigan *et al.*, 1989).

The plasmid DNA content of *A. salmonicida* was investigated by several workers who reported that R-plasmids found in the organism belong to at least three incompatibility (inc) groups. One is the incA group, the second is the incU group and the other is the group of plasmids incapable of stable inheritance in *E. coli*, not belonging to any defined incompatibility groups (Hedges *et al.*, 1985; Brazil *et al.*, 1986; Aoki *et al.*, 1986), these plasmids represent approximately 20% of the *A. salmonicida* cellular DNA (Belland & Trust, 1989). Also, "atypical" strains of *A. salmonicida* showed much greater diversity in plasmid DNA content than the conserved population of plasmids in "typical" strains (Belland & Trust, 1989).

Most of R-plasmids of *A. salmonicida* carry genes encoding resistance to several antibiotics, such as streptomycin, tetracycline, chloramphenicol, sulfadiazine, nalidixic acid, spectinomycin, and trimethoprim (Toranzo *et al.*, 1983; Hedges *et al.*, 1985; Brazil *et al.*, 1986). These resistance determinants were persistent and remained in the natural environment for long periods in the absence of positive selection (Brazil *et al.*, 1986). Aoki *et al.* (1986) isolated and characterized the restriction endonuclease digest patterns of

two related R-plasmids; also, Belland and Trust (1989) analyzed plasmids from "typical" and "atypical" strains of *A. salmonicida* using a minicell expression system in *E. coli* and found that the plasmid DNA of the organism expressed 17 different proteins ranging from 12 kD to 90 kD of which some appeared to be exported proteins. The same authors reported a group of three plasmids ranging from 5-6 kb which were highly conserved, and they found that a chloramphenicol acyltransferase gene was carried by a high M.W. non-conjugative plasmid.

Experiments showing correlations between virulence and plasmids revealed that decreased virulence of *A. salmonicida* was not correlated with loss of a plasmid.

2.4 - Cloning of *A. salmonicida* genes

A. salmonicida surface array protein (A-layer) gene was inserted in the bacteriophage λ gt 11 and cloned into *E. coli* by Belland & Trust (1987). Genomic southern analysis and DNA nucleotide sequencing of the 4.0 kb DNA fragment (Figure 5) indicate that the gene was in a single copy on the chromosome located on a *Hind* III - *Pst* I fragment. These studies also indicated that mechanisms of rearrangement occurred within the gene involving a deletion of genetic material and this probably explains the molecular mechanisms of the loss of the ability of *A. salmonicida* to produce the A-layer at elevated temperatures (Belland & Trust, 1987).

Chu *et al.* (1991) succeeded in sequencing the A-protein gene as a 4.6 kb DNA fragment termed *vapA*; the gene is the smallest S-layer gene sequenced to date and is unique to *A. salmonicida*, no significant homologies with the genes coding for morphologically similar tetragonal surface arrays produced by strains of *A. hydrophila* and *A. sobria*. These authors reported that the gene was difficult to clone because of two inverted repeats in the sequence, which are involved in a cross-over resulting in deletion of almost half of the gene. They also reported that in *E. coli* the cloned gene expressed the A-protein but ^{that it} was not exported suggesting that *A. salmonicida* requires at least one additional gene product for translocation of A-protein across the outer membrane.

Figure 5: The restriction map of a 4.0 kb DNA fragment of *A. salmonicida* cloned into the bacteriophage λ gt 11. The shaded segment corresponds to the A-protein gene sequences (Belland & Trust, 1987).

The A-protein gene:

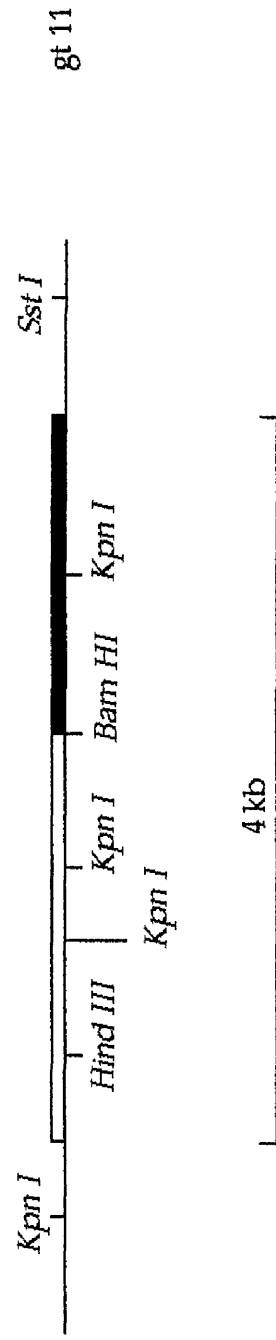
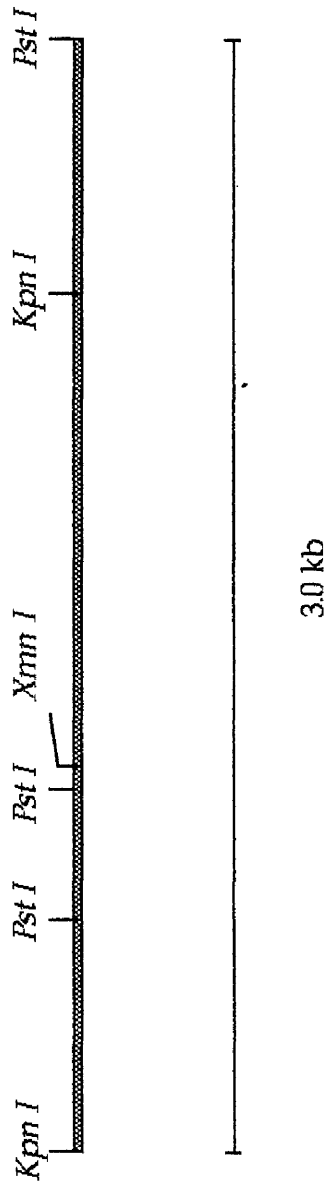


Figure 6: The restriction map of the 3.0 kb DNA fragment encoding the serine protease of *A.salmonicida* (Whitby *et al.* 1992).(Reproduced by kind permission of publishers).

The serine protease gene.



Recently Whitby *et al.* (1992) succeeded in cloning and sequencing a second gene of *A. salmonicida* (Figure 6); they cloned the gene (*aspA*) of the serine protease into phagemid pTZ18R in two restriction fragments, 2.0 kb *Pst* I and 2.3 kb *Kpn* I, of genomic DNA. An open reading frame of 1,863 bp translated into a sequence of 621 amino acids of which 24 amino acids correspond to a signal peptide and 597 amino acids to the mature enzyme of molecular weight 64,173 d. In a very recent report Hirono and Aoki (1993) cloned three different haemolysin genes designated ASH1, ASH3 and ASH4 from *A. salmonicida* into *E. coli*. Also Dodsworth *et al.* (1993) cloned the gene for the maltose-inducible porin (maltoporin) of the organism, they found that the gene encoded for a 420 amino acid mature protein of molecular weight 46424 Da which showed a high degree of similarity with *E. coli* LamB.

Object of research

The main objectives of this study were :

- i)- To generate transposon mutants of *A. salmonicida*.
- ii)- To analyse mutants to characterize the difference in extracellular products and outer membrane proteins profiles.
- iii)- To compare *in vitro* the activities of the different virulence determinants of mutants to their parental strain.
- iv)- To correlate differences in secretion of extracellular products or outer membrane proteins with differences in behaviour in fish.

Materials & Methods

3.1 - Bacterial strains

3.1.1 - *A. salmonicida* strains

A. salmonicida strains used in this study are listed in table 5. Strains 80628 and 4700 were fresh isolates from outbreaks of furunculosis in Scotland. *A. salmonicida* 80550 is an avirulent strain which did not produce the A-layer; this was obtained after repeated passage on agar (Table 5). All these strains were obtained from the Microbiology department culture collection (Glasgow University).

3.1.2 - *E. coli* bacterial strains

The different strains of *E. coli* used in this study and their characteristics are listed in Table 6.

3.2 - Growth conditions and media

Both *A. salmonicida* and *E. coli* were cultivated in Brain-Heart infusion media (BHI, Oxoid). When cultivating bacteria in BHI broth, cultures were agitated in an orbital shaker at 130 rpm for *A. salmonicida* strains and 100 rpm for *E. coli* strains. Dimpled conical flasks were used for bacteria broth cultures.

Bacterial cultures of *A. salmonicida* were incubated at 22 °C for 48 h, and *E. coli* strains were incubated at 37 °C for 24 h.

3.3 - Maintenance of bacterial cultures

Organisms were maintained on BHI agar slopes, subcultured monthly on fresh BHI agar and stored at 4 °C. For long storage, bacterial strains were maintained in 25 % (v/v) glycerol and kept at -20 °C. Organisms were routinely checked for purity by gram staining.

Table 5: Characteristics of *A. salmonicida* strains used in this study.

Strain	resistance marker	presence of A-layer	Autoagglutination in saline
<i>A. salmonicida</i> 80628	furazolidone	+	+
<i>A. salmonicida</i> 4700	nalidixic acid	+	+
<i>A. salmonicida</i> 80550		-	-
<i>A. salmonicida</i> FCC		+	+

Table 6: Characteristics of *E. coli* strains used.

Strain	Relevant characteristics	Plasmid	Source
<i>E. coli</i> HB101	<i>pro</i> -, <i>Sm</i> ^R	pRK2013	MDCC*
<i>E. coli</i> HB101	<i>pro</i> -, <i>Sm</i> ^R	pUW964	"
<i>E. coli</i> WA803	<i>met</i> -, <i>thi</i> -	pGS9	"
<i>E. coli</i> S17-1	<i>pro</i> -, <i>thi</i> -, mobilization strain, RP4-2::Mu-Km:Tn7 Tp ^R , Sm ^R .	pSUP202-1	"
<i>E. coli</i> C600	<i>leu</i> -, <i>thi</i> -, <i>thr</i> -	pLG221	"
<i>E. coli</i> BRD 327 (SM 10)	<i>thi</i> -, <i>thr</i> -, <i>leu</i> -, <i>tonA</i> , <i>lacY supE recA</i> ::RP4-2-Tc::Mu:: <i>λpir</i> .	pRT733	"
<i>E. coli</i> H205 (EMG9)	LacI-	pGLW50	Dr.I. Hunter Department of Genetics
Glasgow Univ			

* : Microbiology Department Culture Collection (Glasgow University).

3.4 - Bacteriological methods

3.4.1 - Filter conjugation

Plasmid transfer was performed using the filter conjugation method of Maniatis *et al.* (1982) to introduce transposons into *A. salmonicida* recipients from different donor strains of *E. coli* carrying a variety of transposon delivery systems (Table 6 and 7).

A. salmonicida strains were cultivated on BHI agar plates at 22 °C for 48 h, and subcultured into 50 ml of BHI broth containing appropriate antibiotics; the flasks were incubated at 22°C overnight in an orbital shaker (130 rpm).

E. coli donor strains were grown on BHI agar plates containing kanamycin (25 µg/ml (w/v)) at 37 °C overnight; the cultures were then used to inoculate flasks containing 50 ml of BHI broth supplemented with kanamycin (25 µg/ml). Flasks were incubated at 37 °C overnight with shaking (100 rpm). Optical density readings of both donor and recipient strains were recorded every hour until each had reached approximately OD₅₄₀=0.4, then appropriate dilutions of each bacterial strain in sterile saline were plated out on BHI agar plates to obtain a viable count.

Cell suspensions at OD₅₄₀ = 0.4 (giving approximately 10⁷ cells ml⁻¹) were diluted in BHI broth (10⁻¹), and 1 ml of diluted *A. salmonicida* suspension was added to 4 ml of diluted *E. coli* suspension, vortexed and using a sterile syringe, 2.5 ml of mixture was filtered through a 2 cm diameter nitrocellulose filter (Whatman filter, pore size 0.45 µm) in a presterilized filter holder. The filters were removed aseptically, placed on the surface of BHI agar plates and incubated at 22 °C for 8 h to overnight. 2.5 ml of *A. salmonicida* suspension was filtered as above for determining the frequency of spontaneous resistance to kanamycin.

The growth on filters was resuspended in 2.5 ml of sterile BHI broth and 100 µl samples of mixtures were spread on selection plates containing appropriate antibiotics; dilutions were made and plated out on BHI agar plates containing appropriate antibiotics for final viable counts for both donor and recipient strains.

Table 7: Relevant characteristics of transposon delivery vectors used.

Plasmid	size (kb)	Relevant characteristics	Reference
pRK2013	48.0	P-group <i>tra</i> genes <i>col</i> E1 replicon mobilization vector, Km ^R .	Figurski & Helinski (1979)
pUW964	73.0	pRK2013 Km ^R ::Tn7 <i>xyz</i> ::Tn5, Tn ^R , Sp ^R , Km ^R .	Weiss <i>et al.</i> (1983)
pGS9	30.5	N-group <i>tra</i> genes p15A replicon Tn5, Cm ^R , Km ^R .	Selvaraj & Iyer (1983)
pSUP202-1	13.2	pBR325 replicon Mob ⁺ , Tn5, Ap ^R , Cm ^R , Km ^R .	Simon <i>et al.</i> (1983)
pGL221	99.0	Col1b <i>drd</i> -1 <i>cib</i> ::Tn5 Km ^R .	Chatfield <i>et al.</i> (1982)
pRT733	ND	pJM103.1 derivative Km ^R , Tn ^{phoA} , <i>ori</i> RBK, <i>mob</i> ⁺ for RP4	Miller & Mekalanos (1988)
pGLW50	10.2	derivative of plasmid pIJ486, Km ^R , provided by Dr. I. Hunter, (Genetics Department) Tn5.	Ward <i>et al.</i> (1986)

A. salmonicida caseinase-negative and kanamycin-resistant transconjugants were picked from selection plates and streaked onto selection plates for a second time. Mutants were checked for purity by gram staining and brown pigment production on BHI agar plates, and stored at -20 °C in 25% glycerol solution.

3.4.2 -Antibiotic-resistance screening

A. salmonicida transconjugants were screened for maintenance and expression of transposons by multi-point inoculation from microtitre trays onto antibiotic containing plates. Transconjugants were inoculated into microtitre trays containing BHI broth and incubated at 22 °C for 48 h; the multipoint inoculator was alcohol flamed to prevent contamination. Both donor and recipient strains were also spotted onto both BHI agar plates and antibiotic-containing plates as controls; transconjugants were also spotted onto BHI agar plates to show growth in the absence of antibiotics.

3.5 -Measurement of bacterial growth

Measurement of bacterial growth was carried out using a Shimadzu U.V-240 spectrophotometer and growth was estimated by measuring the OD₅₄₀ of samples withdrawn at 2 h intervals; when OD₅₄₀ exceeded 1.00 the culture was diluted with sterile BHI broth to give an absorption value within the range of 0.5 - 1.00; sterile BHI broth was used as a blank.

3.6 - Protein estimation

Protein estimation was carried out by the Lowry-Folin method (1951); bovine serum albumin (BSA, Sigma) was used as standard.

3.7 - Autoagglutination test

A. salmonicida mutants were scraped from BHI agar plates and suspended on a clean, grease-free slide, two small drops of sterile saline were added to each sample, *A. salmonicida* 80550 was used as negative control. Samples were observed under the microscope; a positive autoagglutination test gave large “granules” or aggregates of bacteria.

3.8 - Phage sensitivity test of *A. salmonicida* TnphoA mutants

A. salmonicida transconjugants were tested for their sensitivity to three different phages, specific to *A. salmonicida* LPS, which have been isolated in this department; phages Φ_{1A} , Φ_2 and Φ_{13} were provided in SM buffer (Appendix IV) at approximate concentrations of 10^8 - 10^9 p.f.u.ml⁻¹, dilutions were made in SM buffer to give approximately 300 pfu.ml⁻¹.

100 μ l of overnight cultures of mutants grown at 22 °C with shaking in BHI broth, were mixed with 10 μ l of each phage suspension and mixtures were left at room temperature for 30 min to allow adsorption of phages onto bacterial cells; 3 ml of top BHI agar, kept at 45°C, was added to each mixture, mixed, immediately poured onto BHI agar plates and spread. Plates were incubated at 22 °C for 24 h.

3.9 - Preparation of agar plates

3.9.1 - Congo red agar plates

Congo red (Sigma) 1 % (w/v), was dissolved in sterile distilled water and sterilized by autoclaving. The solution was added to BHI agar to a final concentration of 0.01 % (w/v) and poured into sterile petri dishes, allowed to dry at room temperature overnight and stored at 4 °C until required.

3.9.2 - Egg-yolk agar plates

Egg-yolk suspension was prepared by homogenising the yolk from a fresh egg in 20 ml of sterile saline and centrifugation at 2000 rpm for 5min at room temperature to remove large particles. 5 ml of suspension was mixed with 95 ml of BHI agar to give a suspension of 5 % (v/v) of egg-yolk. The mixture was poured into sterile petri dishes, dried and stored at 4 °C until required.

3.9.3 - Coomassie brilliant blue agar plates

Coomassie brilliant blue agar plates were prepared by adding a filter sterilized solution of Coomassie brilliant blue (BDH) to BHI agar at 45 °C, to a final concentration of 0.01 % (w/v). Plates were air dried and immediately inoculated with bacteria.

3.9.4 - Preparation of "sandwich" plates

"Sandwich" plates were prepared by overlaying BHI agar plates, previously inoculated with mutants, with a 5 % suspension of salmon red blood cells (RBCs) prepared in 2 % (w/v) BHI agarose. Fresh salmon blood was washed three times in sterile PBS and a 20 % suspension in the same buffer was prepared; from this stock suspension, 5 % salmon blood in 2 % low gelling point agarose (Sigma) was prepared and kept at 37 °C. Kanamycin containing BHI agar plates, supporting growth of transconjugants, were overlayed with the blood suspension, and stored in the cold room (4°C) to set for 2 h and then exposed to UV radiation for 5 min. These plates were incubated at 22 °C for 24 h.

3.9.5 - Horse blood agar plates

Fresh horse blood was washed three times in sterile PBS and resuspended in the same volume of PBS. 7 % (v/v) horse blood-BHI agar suspension was prepared, poured into sterile petri dishes and the plates were exposed to UV radiation for 5 min. These plates were immediately used for haemolysis screening of transconjugants.

3.9.6 - Selection plates

Selection plates were prepared by adding the appropriate antibiotics to BHI agar. Kanamycin (Sigma) was added at a concentration of 25 µg/ml to BHI agar containing 80 µg/ml (w/v) of furazolidone (Sigma) and 1 % (w/v) of casein (milk powder, Marvel). These plates were used for screening *A. salmonicida* 80628 transconjugants.

Nalidixic acid (Sigma) and BCIP (5-bromo-4-chloro-3-indoyl phosphate, Sigma) were added to BHI agar to final concentrations of 30 µg/ml (w/v) and 20 µg/ml (w/v).

respectively, and casein to 1 % (w/v), those plates were used to select mutants of *A. salmonicida* 4700 carrying transposon TnphoA.

3.10 - Biochemical Assays

3.10.1 - Caseinase assay

The assay was carried out using azocasein (Sigma) as substrate for caseinase; test samples were prepared by adding 100 μ l of culture supernatant to 100 μ l of BHI broth and 100 μ l of 1 % (w/v) azocasein in 0.1 M phosphate buffer pH 7.5 in sterile microfuge tubes. The contents were mixed, tubes were incubated at 37 °C for 30 min in a temperature-controlled water bath, after which time 800 μ l of 5 % (w/v) cold trichloroacetic acid (Sigma) was added to stop the reaction. The mixtures were centrifuged at 13,000 rpm for 15 min at room temperature using a Heraeus Sepatech Biofuge, pellets were discarded and 800 μ l of supernatants were transferred into fresh microfuge tubes containing 800 μ l of 0.5 N NaOH and mixed to develop the red colour. OD readings were carried out in Shimadzu UV-240 spectrophotometer; the blank was prepared by adding an equivalent volume of sterile BHI broth instead of culture supernatant and was heated at 80 °C for 10 min to inactivate any protease activity. Absorption readings were at 440 nm and one protease unit was defined as the amount of enzyme which caused an increase of 0.1 OD₄₄₀ unit under the above conditions.

3.10.2 - Conductimetric enzyme assay of the phospholipase activity of the GCAT.

Assay of GCAT activity was performed using the conductimetric enzyme assay (Lawrence, 1971), which measures the change in conductance, between two electrodes, due to the activity of GCAT acting upon the substrate. Culture supernatants (48 h) of mutants were used as crude enzyme preparations, dioctanoylphosphatidylcholine (DPC, 40 mg/ml provided by Dr. T.H. Birkbeck), was used as the substrate; the assay was carried out in 10 mM triethanolamine-HCl buffer pH 8.0 ; cells of the apparatus were washed three times with 70 % (v/v) ethanol and rinsed with sterile distilled water, and then rinsed with buffer, 2 ml of buffer was loaded into each cell and left for about 5 min. to achieve thermal

equilibration, then 2 μ l of DPC substrate solution were added. Immediately, 40 μ l of fresh culture supernatant of each mutant was loaded into each cell and results were recorded, using a dedicated computer, as percentages of conductance change and were expressed as μ M of fatty acid released using standard curves already established for the experiment.

In order to differentiate the GCAT and phospholipase C activities which could be contained in culture supernatants of *A. salmonicida*, an inhibition test using EDTA and a stimulation test using CaCl_2 were carried out by the method described above. 2 μ l of DCP was added to 2 ml triethanolamine buffer pH 8.0, 5 μ l (1 mg/ml) of commercial phospholipase C (type IX *C.perfringens*, Sigma) was added and reaction was left to proceed for few seconds; 20 μ l (100 mM) of CaCl_2 (Sigma) was added to induce stimulation, similarly 20 μ l (100 mM) of EDTA was added to another cell of the apparatus containing equivalent ingredients, to inhibit the activity of phospholipase C. Similar tests were performed on culture supernatant of *A. salmonicida* 4700 to determine whether the bacterium produced GCAT and/or phospholipase C in culture supernatants.

3.11 - Haemolytic titration

The haemolytic titre of T-lysin was determined by making serial doubling dilutions of culture supernatants of mutants. Fresh salmon blood was washed three times in sterile phosphate-buffered saline (PBS) pH 7.2 and resuspended to 2 % (v/v) in PBS. 100 μ l of culture supernatants of each mutant were diluted in twofold steps in PBS using microtitre plates, then 100 μ l of 2 % blood suspension were added to each well and plates were incubated at 22 °C overnight (\approx 18 h); haemolytic titres were defined as the dilution factors which gave visibly complete lysis of salmon blood.

3.12 - SDS-polyacrylamide gel electrophoresis of proteins

The method was based on those described by Laemmli (1970) and Ames (1974) using a vertical slab gel tank. The stock solutions and recipes for gel and buffer preparation are shown in appendix III.

Separating (lower) and stacking (upper) gels contained 12.5 % (w/v) acrylamide. The gel was formed between two glass plates of 17 cm x 19 cm x 0.3 cm with spacers of 1.5 mm thick each. Gel and electrophoresis (running) buffers contained 0.1 % (w/v) SDS.

3.12.1 - Preparation of samples for SDS-PAGE

Concentrations of cell envelope preparations were adjusted to 0.5 - 1.0 mg/ml of proteins by the Lowry-Folin method; whole cells and culture supernatants preparations were not diluted. Samples were collected in sterile microfuge tubes and equal volumes of appropriate solubilizing buffer (see Appendix III) were added to each samples and heated at 100°C for 3 min in a boiling water bath.

To estimate the molecular weight of separated polypeptides, a mixture of polypeptides of known molecular weight was used (SDS-7, Sigma); this contained a mixture of the following seven proteins : α -lactalbumin (14,200 Da) , trypsin inhibitor (20,100 Da), trypsinogen (24,000 Da) , carbonic anhydrase (29,000 Da) , glyceraldehyde-3-phosphate dehydrogenase (36,000 Da) , egg albumin (45,000 Da) , and bovine albumin (66,000 Da). Protein markers were also incubated at 100 °C for 3 min. prior to electrophoresis.

3.12.2 - Preparation of samples for gelatin-PAGE

24 h bacterial cell cultures grown in BHI broth at 22 °C with shaking were used ; culture supernatants were obtained by centrifugation of culture samples at 10,000 rpm for 10 min. at room temperature, 100 μ l of culture supernatant of each organism were added to the following mixture contained in sterile microfuge tubes : 10 μ l of 25 % (w/v) SDS (Sigma), 10 μ l of 40 % (v/v) glycerol and one drop of a 0.001% solution of bromophenol blue (Sigma). Samples were mixed and immediately loaded onto gels.

3.12.3 - Electrophoresis of protein samples

30 μ l samples were loaded onto gel and electrophoresis was performed at room temperature at a constant current of 30 mA, using a Shandon VoKam SAE 2761 power

unit, until the tracking dye had reached the bottom of the gel. Where subsequent analysis of samples by immunoblotting was required, duplicate samples were loaded onto each half of the gel. Following electrophoresis, one half was stained using an appropriate staining method and the other was subjected to western blotting.

3.13 - Analysis of gels after electrophoresis

3.13.1 - Coomassie blue staining

Gels were stained using the method of Weber and Osborn (1969). Gels were soaked in Coomassie blue staining solution (see Appendix III) for 3 h. using Coomassie blue stain R 250 (BDH) at room temperature, then destained the following day in destaining solution (see Appendix III) with three changes of solution. Finally, gels were soaked in distilled water and stored at 4 °C in heat-sealed cellophane bags.

3.13.2 - Silver staining of proteins

This method was adapted and modified from the procedure of Oakley *et al.* (1980). Gels were prefixed in a solution containing 50 % (v/v) absolute alcohol and 10 % (v/v) acetic acid in distilled water, overnight at room temperature; the fixing solution was removed and replaced by a solution containing 10 % (v/v) glutaraldehyde (BDH) in distilled water. After a gentle shaking for 30 min gels were then rinsed in a large volume of distilled water overnight, then in fresh distilled water for 30 min. Gels were soaked in a freshly prepared solution containing 5 µg/ml of dithiothreitol (Sigma) in distilled water for 30 min, the solution discarded and replaced by 0.1 % (w/v) silver nitrate solution (BDH) for 30 min. Gels were rinsed once in distilled water and then twice rapidly in developer (50 µl of 37 % formaldehyde in 100 ml of 3 % (w/v) sodium carbonate solution) until the desired level of staining was reached, then 5 ml of 2.3 M citric acid solution was added to developer and mixed for 10 min. Finally, gels were soaked in 0.03 % (w/v) sodium carbonate solution for 10 min. and stored at 4 °C in heat-sealed cellophane bags.

3.13.3 - Silver staining of LPS

LPS of *A. salmonicida* was stained using the method of Tsai and Frasch (1982). Following electrophoresis, gels were fixed in 40 % ethanol - 5% acetic acid solution overnight at room temperature. The fixing solution was replaced with 0.7 % (w/v) periodic acid in fixing solution for 5 min to oxidize LPS, a further three 15-min washes in distilled water were performed and the gel was stained for 10 min with gentle agitation in staining reagent (containing 2 ml of concentrated ammonium hydroxide added to 28 ml of 0.1 N sodium hydroxide); 5 ml of 20 % (w/v) silver nitrate (BDH) was added immediately before use, and 115 ml of distilled water. The gel was washed three times in distilled water for 30 min and developed by adding 200 ml of developer (200 ml of distilled water containing 50 mg of citric acid and 5 ml of 37 % formaldehyde) for 3 to 5 min. When the desired level of staining was reached the gels were immediately washed in distilled water with several changes and stored in heat sealed cellophane bags.

3.13.4 - Staining of gelatin gels

Following electrophoresis, gels were washed in a 2.5 % (v/v) Triton X 100 (Sigma) solution for 30 min, followed by incubation of the gel at 37 °C in 0.1 M glycine (Sigma) pH 7.5 for 45 min. Gels were fixed and stained overnight in 0.1 % (w/v) amido black solution (Sigma). Finally, gels were destained in a mixture of methanol / acetic acid / distilled water (1/1/1, v/v/v) throughout the day with several changes, then rinsed with distilled water and heat sealed in cellophane bags.

3.14 - Partial peptide mapping of the A-protein

In order to determine the nature of protein bands visualized on SDS-PAGE, one-dimensional peptide mapping was performed using the method of Perera *et al.* (1985). Gel slices were treated with N-chlorosuccinimide (NCS, Sigma) in urea using the method of Lischwe and Ochs (1982), gel slices were washed first with 20 ml of distilled water for 20 min. with one change and then with 10 ml of urea / H₂O / CH₃COOH (1/1/1, w/v/v) for 20

min. with one change. The standard cleavage conditions were carried out for 30 min. in 0.015 M NCS in 5 ml of urea / H₂O / CH₃COOH solution. The slices were washed with distilled water as above and equilibrated in 10 % (v/v) glycerol, 15 % (v/v) β-mercaptoethanol, 3 % (w/v) SDS and 0.06 M Tris-HCl pH 8.0 (10 ml) solution for 90 min, with three changes; finally, slices were inserted into the wells of a 4 % (w/v) acrylamide stacking gel, and peptides were separated in a 17 % (w/v) acrylamide resolving gel. Untreated slices were also electrophoresed in parallel for comparison. The gels were stained with silver according to the method described by Oakley *et al.* (1980) (see section 2.14.2).

3.15 - Immunological techniques

3.15.1 -Western blotting

Protein transfer from SDS-PAGE onto nitrocellulose filters (Hybond C, Amersham) was carried out according to the method of Towbin *et al.* (1979) except that 80 mA constant current was applied to electroblot the membrane overnight following electrophoresis using a 12 % acrylamide gel. "Sandwiches" were assembled between single layers of 3 MM thick filter paper (Whatman) and transfers were carried out in a pre-cooled transfer buffer (see Appendix IV) at 80 mA overnight with cooling in a Bio-Rad "Trans-blot" transfer apparatus. Following transfer, blots were soaked in TTS buffer (see Appendix IV) overnight and then in TTS buffer containing 2 % (w/v) bovine serum albumin (BSA, Sigma) for 1 h; filters were immersed in TTS buffer containing appropriate volumes of antiserum* (100 µl-200µl) for 90 min, washed 5 times in fresh TTS buffer for 45 min and immersed in fresh TTS buffer containing 1/500 anti-rabbit horse radish peroxidase conjugate (HRP) (Scottish Antibody Production Unit) and incubated for 90 min at room temperature. Filters were washed in TBS buffer (TTS buffer without Tween 20) for 10 min and then in TBS containing 0.05 % (v/v) Nonidet P40 for 10 min and washed once in TBS only for 10 min. The substrate (3,3 Diaminobenzidine tetrahydrochloride dihydrate, Aldrich Chemical Co.) (Appendix IV) was added and when the desired level of staining had been reached the

* Antiserum to extracellular products of *A. salmonicida* 80628 was raised in rabbits which were injected with 1 ml of emulsion of equal volumes of 48 h culture supernatant and complete Freund's adjuvant. Further injections of culture supernatant in incomplete Freund's adjuvant were carried out on days 14 and 44 and rabbits were bled 7 days later and the sera obtained.

reaction was stopped by immersing the developed filters in distilled water for 10 min. Finally, filters were rinsed in distilled water, dried and stored.

3.15.2 - Ouchterlony gel diffusion test

The procedure was adapted from the method described by Ouchterlony (1958). 1 % (w/v) agarose (type I : low EEO, sigma) in Tris-HCl buffer pH 7.5 was prepared and poured onto microscope slides. Samples were applied to 3 mm diameter wells and slides were incubated in a moist atmosphere at 37 °C overnight. Slides were washed at room temperature in sterile saline for 1 day with three changes of solution, rinsed in distilled water, pressed, air-dried and stained with Crowle and Kline stain for 10 min; slides were destained in 3 % (v/v) acetic acid until the precipitin lines were clearly visible, washed in distilled water, and then heat-sealed in cellophane bags.

3.16 - DNA analysis techniques

3.16.1 - Agarose gel electrophoresis of DNA

Agarose gel electrophoresis of DNA was performed using a flat-bed apparatus (Pharmacia, LKB). Agarose, 0.7 % (type II-A : Medium EEO, Sigma) was dissolved in 0.5 x TBE buffer (Appendix II) by boiling, the molten agarose was cooled to 50 °C and poured into a horizontal gel mould (8 cm x 10.5 cm for 50 ml of agarose, or 20 cm x 20 cm for 200 ml of agarose) and allowed to solidify with the appropriate comb in place. The gel mould was placed in the electrophoresis tank and immersed in 0.5 x TBE running buffer. DNA samples containing 1/10th volume of 10 x gel loading buffer (60 % (w/v) sucrose, 0.1 % (w/v) bromophenol blue in distilled water) were loaded into the wells. The 50 ml gels were usually run at 100 V for 2 h, and gels of 200 ml were usually run at 40 V overnight. Following electrophoresis, gels were soaked in a solution of ethidium bromide (25 µl of 10 mg/ml solution in 500 ml of distilled water, Sigma) for 30 min, then soaked in distilled water for 10 min to remove the excess ethidium bromide. DNA bands were

visualised by illumination with short wave ultraviolet light, and photographed through a red filter using Polaroid type 667 film.

When low melting-point agarose (type I : low EEO, Sigma) was used, gels were prepared in the same way as described above except that gels were allowed to set in the cold room (4 °C) and electrophoresis was also carried out at 4 °C.

3.16.2 - Plasmid DNA preparations

3.16.2.1 - Kado and Liu procedure

Plasmid DNA were extracted using a modification of the method of Kado and Liu (1981). Overnight cultures of bacterial cells, grown at 22 °C with shaking in 100 ml of BHI broth containing 25 µg/ml kanamycin, were used. Using sterile microfuge tubes, 1.5 ml of each culture was harvested by centrifugation at 13,000 rpm for 5 min at room temperature. Pellets were resuspended in 100 µl of lysis buffer (Appendix II), then mixed and incubated at 60 °C for 45 min in a water bath. Plasmid DNA was extracted with 200 µl of phenol : chloroform mixture (1:1, v/v), the emulsion was separated by centrifugation at 13,000 rpm for 10 min at room temperature, and the top aqueous layers were retained and stored at -20 °C in sterile microfuge tubes.

3.16.2.2 - Plasmid midi preparation

Plasmid DNA were extracted using the Quiagen plasmid midi and maxi preparation kit (Diagen). Bacterial cells were grown at 22 °C with shaking overnight in 150 ml of BHI broth containing 25 µg/ml kanamycin. Plasmid DNA was extracted following the manufacturer's instructions; final pellets were resuspended in 50 µl of sterile deionized water and kept in microfuge tubes at -20 °C until required.

3.16.3 - Miniprep of bacterial genomic DNA

Extraction of bacterial genomic DNA was performed using a method adapted from Ausubel *et al.* (1987). Cultures of *A. salmonicida* mutants in BHI broth containing 50

$\mu\text{g/ml}$ Km (24 h) were used; 1.5 ml of each culture was harvested by centrifugation at 10,000 rpm for 2 min at room temperature in microfuge tubes. Pellets were resuspended in 567 μl of TE buffer (Appendix II), 30 μl of 10 % (w/v) SDS and 3 μl of 20 mg/ml proteinase K (Sigma) were added to give a final concentration of 100 $\mu\text{g/ml}$ proteinase K in 0.5 % (w/v) SDS. Mixtures were vortexed thoroughly and incubated at 37 °C for 1 h 100 μl of 5 M NaCl was added and mixed thoroughly, then 80 μl of CTAB / NaCl solution (see Appendix I) was added, mixed thoroughly and incubated at 65 °C for 10 min in a water bath. An approximately equal volume (750 μl) of chloroform / isoamyl alcohol (24:1, v/v) was added, and mixed thoroughly. Mixtures were centrifuged at 13,000 rpm for 5 min at room temperature, then aqueous supernatants were transferred into fresh microfuge tubes and an equal volume of phenol / chloroform / isoamyl alcohol (25/24/1) was added, mixed and centrifuged at 13,000 rpm for 5 min at room temperature. Supernatants were transferred into fresh microfuge tubes and nucleic acids were precipitated by adding 0.6 vol of isopropanol and centrifuged at 5,000 for 30 sec at room temperature. Pellets were washed with 70 % (v/v) ethanol and supernatants were removed, the pellets were air dried, resuspended in 100 μl of sterile deionized water and stored at -20 °C until required.

3.16.4 - Southern blotting and hybridization

Restriction enzyme digests of *A. salmonicida* chromosomal DNA were transferred to nylon membrane (Hybond-NTM, Amersham) using the method of Southern (1975). Following electrophoresis, the gel was placed in a solution of 0.2 N HCl for 10 min, the solution was replaced with 500 ml of denaturation solution (Appendix II) for 15 min, a second step using fresh denaturation solution was performed, then the gel was placed in 500 ml of neutralization solution (Appendix II) for 30 min. The nylon membrane was wetted in water and immersed in 20 x SSC for 10 min and the gel placed on the transfer pyramid already prepared according to the protocol. Air bubbles were removed between the gel and wick, which was wetted in 20 x SSC with its ends hanging over the glass plate into the 20 x SSC; the nylon membrane was placed on the gel and the transfer pyramid was set

up and pressed down by a weight (≈ 0.5 kg) overnight. After transfer, the nylon membrane was washed in 2 x SSC for 5 min, then blotted between Whatman 3 MM filter paper and wrapped in Saran Wrap™ (Dow Chemical Co.). The damp filter was examined under UV illumination to show transfer and to fix the DNA on the membrane filter and then stored at 4 °C.

3.16.5 - Hybridization conditions

The filter was heat-sealed in plastic bag containing 12 ml of prehybridization solution (Appendix II) and incubated at 42 °C overnight in a water bath with gentle shaking. The prehybridization solution was removed and 40 μ l of labelled DNA probe were added to 12 ml of hybridization solution (Appendix II), the bag re-sealed and incubated at 42 °C overnight.

The filter was removed, immediately washed in a solution of 2 x SSC, 0.1 % (w/v) SDS at room temperature for 30 min with gentle agitation, and then washed in 1 x SSC, 0.1 % (w/v) SDS at 65 °C for 2 h. It was finally wrapped damp in Saran Wrap and exposed to X-ray film (X-OMAT-S, Kodak) at -70 °C for 3 days.

3.16.6 - Restriction endonuclease digestion

EcoR I and *Xho* I restriction endonucleases and 10 x reaction buffers were purchased from Gibco, BRL, Paisley, Scotland. Enzymes were used according to the manufacturer's instructions. Reactions were carried out overnight at 37 °C, and 5 μ l of loading buffer (Appendix II) were added to 20 μ l of digested DNA before loading onto a gel.

3.16.7 - Preparation of restriction fragment probe

Restriction fragment of plasmid pGLW50 digested with *Xho* I were separated in 1 % (w/v) of low-gelling temperature agarose (Sigma). After electrophoresis, the gel was stained with ethidium bromide and the desired band was excised under UV illumination. The probe was extracted and purified from gel slices using Gene Clean II kit (Diagen) and stored at -20 °C until required.

3.16.8 - Random hexanucleotide labelling of probes

End-labelling of the probe was carried as follows :

34 μ l of DNA probe sample was denatured by boiling in water for 5 min and immediately cooled on ice, 10 μ l of Oligo-Labelling Buffer (OLB, obtained from M.Ward), 2 μ l of 10 mg/ml BSA (Gibco, BRL), 2 μ l of α -³²P-dATP (10 μ Ci/ μ l, Amersham), and 2 μ l of Klenow fragment of DNA polymerase I (Boehringer Mannheim) were mixed and incubated at room temperature overnight. The mixture was boiled in water for 5 min, cooled on ice and immediately used for southern blot hybridization.

3.16.9 - Estimation of DNA molecular size

Lambda DNA (BRL) was cleaved with *Hind* III to yield fragments of the following sizes : 23.13 kb, 9.416 kb, 6.556 kb, 4.361kb, 2.322 kb, 2.027 kb, 0.564 kb and 0.125 kb.

A graphical relationship was established between molecular size and mobility by plotting the log₁₀ of DNA fragment length versus the distance migrated from the origin. Estimation of plasmid DNA size was carried out using the method of Platt and Taggart (1987).

3.17 - LPS extraction

LPS of *A. salmonicida* mutants was extracted using the method of Westphal and Jann (1965). 100 ml of overnight bacterial cell cultures in BHI broth at 22 °C with shaking, were harvested by centrifugation (10,000 rpm for 15 min at 4 °C), pellets were resuspended in 10 ml of hot (68 °C) sterile distilled water and 10 ml of 90 % (w/v) hot phenol (68 °C). Mixtures were incubated at 68 °C for 30 min in a water bath, then cooled to 10 °C and centrifuged at 7,000 rpm for 45 min at 4 °C. After further centrifugation the phases were separated, the phenol layer twice mixed with 10 ml of distilled water and aqueous layers was collected; the combined water extracts were dialyzed against cold tap water for 2 days and against distilled water for 1 day. Samples were then centrifuged at

5,000 rpm for 15 min to remove traces of insoluble material and the supernatants were centrifuged at 40,000 rpm for 3 h at 4 °C; the sedimented gels were resuspended in sterile distilled water, then freeze-dried and stored at 4 °C.

3.18 - Extraction of outer-membrane proteins

One litre 48 h broth cultures of bacterial strains were harvested by centrifugation at 6,000 rpm for 20 min. at 4 °C, the resultant pellets were washed twice in sterile PBS and resuspended in 50 ml of PBS containing 0.1 M phenylmethylsulphonylfluoride (PMSF, Sigma). Cells were broken by a total of six minutes sonication in an MSE Soniprep ultrasonic desintegrator and unbroken cells were removed by centrifugation at 6,000 rpm for 20 min at 4 °C. Five ml of 22 % (w/v) sodium N-laurylsarcosinate (Sigma) was added and mixtures were incubated at 20 °C for 30 min in a water bath ; the outer membrane-peptidoglycan complex was sedimented by centrifugation at 40,000 rpm for 45 min at 4 °C using a Sorval ultracentrifuge (Dupont), pellets were resuspended in 50 ml of 22 % (w/v) sodium N-laurylsarcosinate containing 0.1 mM PMSF and the outer membrane complex again recovered by centrifugation. Then pellets were resuspended in 50 ml of 10 mM Tris-HCl buffer pH 8.0 containing 2 % (w/v) SDS and insoluble material was recovered by centrifugation at 100,000 g for 45 min at 20 °C; pellets were washed once more in the same way. The final ⁱⁿsoluble material, peptidoglycan associated with proteins, was retained, and pellets were resuspended in 50 ml of 10 mM Tris-HCl buffer pH 8.0, 2 % (w/v) SDS and 1 M NaCl and incubated at 37 °C for 90 min in a water bath to release porins and some peptidoglycan-associated lipoproteins. The peptidoglycan residue was removed by centrifugation at 100,000 g for 45 min at 20 °C. Supernatants containing outer membrane proteins were retained and freeze-dried.

3.19 - Electron microscopy of *A. salmonicida* TnphoA mutants

To 30 ml of bacterial suspension, 3 ml of 5 % (v/v) glutaraldehyde in 0.1 M cacodylate buffer were added, cells were harvested by centrifugation (13.000 rpm for 10 min at room temperature) and resuspended in 1 ml of the same buffer in microfuge tubes.

Samples were incubated in the dark for 2 h, cells were washed three times in 0.1 M cacodylate buffer and subjected to a second fixation in 1% (w/v) osmium tetroxide in 0.1 M cacodylate buffer. Cells were washed as previously, pellets resuspended in the second fixation buffer and incubated for 2 h in the dark; cells were then pelleted in the microcentrifuge and washed several times in distilled water. Pellets were then resuspended and treated in 0.5 % (w/v) uranyl acetate for 2 h in the dark, washed several times in distilled water and resuspended in 1.5 % (w/v) molten ion agar; after solidification the agar was cut into small blocks.

The samples contained in blocks were dehydrated with increasing concentration of ethanol (30 %, 50 %, 70% and 100 %), the alcohol was then replaced with epoxyl 1-2 propane for three transfers. Samples were embedded in epoxyl 1-2 propane / araldite (1:1 ratio)

Ultrathin sections were collected on copper grids, stained with aqueous uranyl acetate and lead citrate, and then examined in a Zeiss EM902 electron microscope.

Results

Initially the transposon Tn5, carried on the suicide plasmid pUW 964, was investigated for mutagenesis of *A. salmonicida*. Several strains of *A. salmonicida*, principally field isolates obtained from Marine Harvest Ltd, Lochailort, were considered. Because of the possible problems reported by Wood *et al.* (1986), that induction of resistance to certain antibiotics often led to resistance to a range of antibiotics, changes in OMP profile and expression of protease, a field isolate, strain 80628 with intermediate resistance to furazolidone was chosen.

4.1- Antibiotic sensitivity of mutants generated using suicide plasmid pUW 964

The expression of antibiotic resistance genes of plasmid pUW964, which in addition to transposon Tn5 carries a section of Tn7 encoding resistance to the antibiotics kanamycin (Km), trimethoprim (Tm), spectinomycin (Sp), streptomycin (St) and bleomycin (Bl), was investigated. The expression of these antibiotic markers in the conjugated mutants gives an indication of whether Tn5 and/or Tn7 have been transferred to the recipient. Initially, *E. coli* HB 101 and *A. salmonicida* 80628 were tested for their resistance to these markers (Table 8).

The donor strain *E. coli* HB 101 showed resistance to all transposon markers indicating that plasmid vector pUW964 was maintained and expressed; *A. salmonicida* 80628 was resistant to furazolidone as described previously; however, the sensitivity of this strain was variable when repeatedly tested using the same conditions. On different occasions the strain showed resistance to high concentration of furazolidone ($100 \mu\text{g ml}^{-1}$) but sometimes it showed resistance only to low concentrations of the same antibiotic ($20 \mu\text{g ml}^{-1}$). These variations in resistance were considered to be due to the fact that *A. salmonicida* 80628 may express this resistance at different stages during its growth cycle.

Further investigation of the resistance of *E. coli* and *A. salmonicida* to furazolidone was done with BHI agar plates containing different concentrations of the antibiotic. Growth of the bacteria was inhibited at concentrations of $20 \mu\text{g ml}^{-1}$ for *E. coli*

Table 8 : Sensitivity of *E. coli* HB 101 and *A. salmonicida* 80628 to antibiotic selection markers.

Antibiotics	Concentration ($\mu\text{g.ml}^{-1}$)	<i>E.coli</i> HB 101	<i>A. salmonicida</i> 80628
Trimethoprim	100	R	S
Streptomycin	30	R	R*
Spectinomycin	100	R	R*
Kanamycin	50	R	S
Furazolidone	100	S	R*

R: resistant; S: sensitive.

* : Varied resistance.

HB 101 and at $40 \mu\text{g ml}^{-1}$ for *A. salmonicida* 80628 (Figure 7); when grown in the presence of $20 \mu\text{g ml}^{-1}$ furazolidone, 8.4 % of *E. coli* HB101 cells were viable which represented a very high rate of survival and 80.7 % of *A. salmonicida* 80628 cells were resistant to the same concentration; however approximately 3.4 % of the total cells were resistant to $40 \mu\text{g ml}^{-1}$ of furazolidone.

Filtration of furazolidone solutions was not possible because of the limited solubility of the antibiotic in water. Also, the antibiotic was sensitive to autoclaving at 121°C for 15 min but most activity was retained when the time of autoclaving was reduced from 15 min to 10 min.

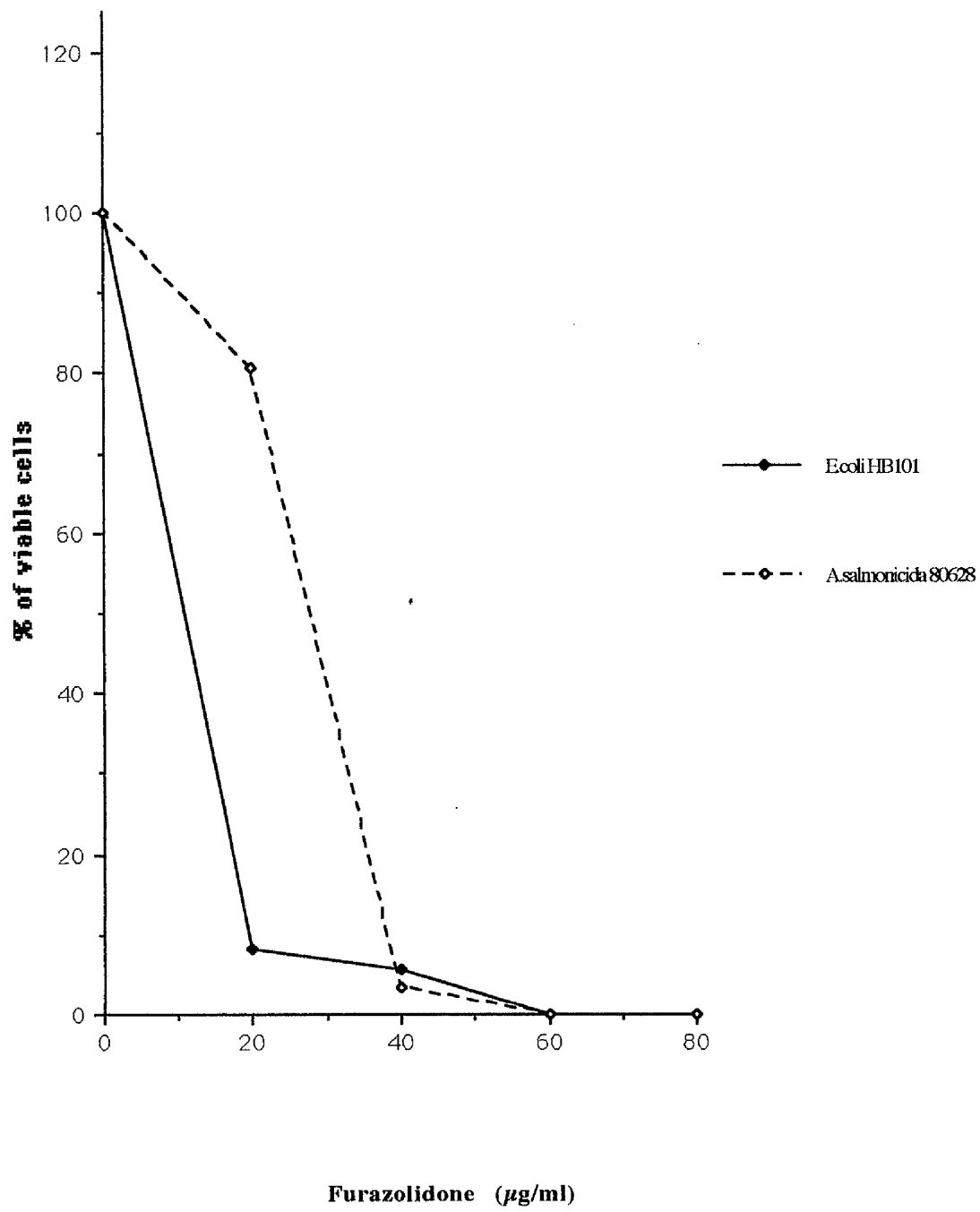
4.2 - Frequencies of transfer of Tn5 plasmid donors

Mutagenesis was initially carried out using *A. salmonicida* strain 80628 as the recipient and *E. coli* as the plasmid donor strain. Since the optimum growth conditions for *A. salmonicida* are different from those of *E. coli*, the conditions of mating between the two strains were optimized by varying the concentrations of both donor and recipient strains, the mating temperature, duration of mating and the medium used for matings.

For all mating experiments, bacteria were subcultured and freshly grown to exponential phase. The concentrations of both donor and recipient bacteria were varied between 2.5×10^7 cells ml^{-1} and 7×10^9 cells ml^{-1} for *A. salmonicida* and between 4.3×10^7 cells ml^{-1} and 1.4×10^9 cells ml^{-1} for *E. coli*. The ratios of *E. coli* / *A. salmonicida* were between 1:1 and 1:4.

A. salmonicida was normally cultured at 20 or 22°C . At 25°C the growth rate was higher than at 22°C and cells reached stationary phase after only 24 h; at 30°C growth was impaired and was considered too high for mating. Although *E. coli* is normally cultivated at 37°C it does grow slowly at 22°C . Thus, a temperature of 22°C was chosen, as this permitted good growth of both strains.

Figure 7 : Viability of *A. salmonicida* 80628 and *E. coli* HB101 in BHI broth. Cultures were incubated at 22 °C and 37 °C for *A. salmonicida* and *E. coli* , respectively, in an orbital shaker; 0.1 ml of dilutions of both cell suspensions in the same medium were spread onto BHI agar plates containing furazolidone, and then incubated at appropriate temperatures.



For various times of mating and antibiotic concentrations, the frequency of transposition achieved and spontaneous mutation frequencies are shown in Tables 9 and 10.

Frequencies of Tn5 insertions were varied, the lowest frequency obtained being approximately 2×10^{-8} and the highest 2.4×10^{-2} , in over 18 matings using *A. salmonicida* 80628 as recipient strain and *E. coli* HB 101 as donor. Nine matings did not show any transconjugants, which was probably due to the selective antibiotics used. As no transconjugants were obtained after matings when mixtures of *E. coli* / *A. salmonicida* were plated on selection plates containing 100, 50 and 25 $\mu\text{g ml}^{-1}$ of furazolidone, it seems that the appearance of mutants on selection plates was considerably affected by the synergistic effect of kanamycin and furazolidone. However, mutants were obtained on selection plates containing as much as 80 $\mu\text{g ml}^{-1}$ furazolidone, perhaps because the antibiotic lost much of its effect on cells after autoclaving. This was observed when BHI agar containing furazolidone was autoclaved at 121 °C for 20 min, but was not observed when medium containing the antibiotic was autoclaved for 10 min at 121 °C (pressure cooker). Thus, it seems that furazolidone was affected by autoclaving but was not completely destroyed because growth of *E. coli* was still inhibited by the antibiotic.

The effect of temperature on plasmid transfer was demonstrated by performing matings at 20, 22 and 25°C; transconjugants were obtained at 20 °C or 22 °C but not at 25 °C. The optimum temperature was 22°C although *E. coli* grew slowly at this temperature. Changing the ratios of *E. coli* to *A. salmonicida* had no apparent effects on the frequency of plasmid transfer. Also, incubating mixtures of *E. coli* and *A. salmonicida* for longer times did not alter the frequency of transfer; however, matings for less than 8 hours did not produce transconjugants.

96 Km^R colonies isolated from various matings were replated on BHI agar plates containing Km (25 $\mu\text{g ml}^{-1}$) to check the stability of their kanamycin-resistance phenotype; 18 colonies failed to grow indicating a loss of expression of kanamycin-resistance which was probably due to excision of Tn5.

Table 9 : Frequency of formation of *A. salmonicida* 80628 Km^R colonies following transposon mutagenesis using *E.coli* HB101 pUW964

Temperature of mating (°C)	Length of mating (h)	Fur ($\mu\text{g ml}^{-1}$)	Km ($\mu\text{g ml}^{-1}$)	Ratio <i>E. coli</i> / <i>A. salmonicida</i>	Frequencies of <i>A. salmonicida</i> Km ^R colonies
20	8	100	100	1:4	$< 0.4 \times 10^{-6} *$
25	18	100	50	1:3	$< 0.32 \times 10^{-7} *$
25	24	100	50	1:2	$< 0.45 \times 10^{-7} *$
25	8	100	50	1:3	$< 0.55 \times 10^{-8} *$
25	24	100	40	1:1	$< 0.21 \times 10^{-7} *$
25	24	100	30	1:1	"
25	24	100	25	1:1	"
20	24	80	25	1:4	2×10^{-8}

Table 10: (continued)

Temperature of mating (°C)	Length of mating (h)	Fur ($\mu\text{g ml}^{-1}$)	Km ($\mu\text{g ml}^{-1}$)	Ratio <i>E. coli</i> / <i>A. salmonicida</i>	Frequencies of <i>A. salmonicida</i> Km ^R colonies
22	18	80	50	1:1	3.8×10^{-5}
22	18	25	50	1:2	$< 0.5 \times 10^{-8} *$
22	18	20	25	1:1	2.4×10^{-2}
22	18	20	25	1:1	1.3×10^{-5}
22	18	20	25	1:1	7.6×10^{-4}
22	96	20	25	1:2	1.4×10^{-5}
22	18	20	25	1:1	3.3×10^{-5}
22	18	50	50	1:1	$< 0.17 \times 10^{-8} *$

*: No transconjugants obtained from matings; Fur : furazolidone; Km : kanamycin.

4.3 - Spontaneous kanamycin-resistance in *A. salmonicida* 80628

Frequencies of spontaneous kanamycin-resistance in strain 80628 were found to be in the range of 3.8×10^{-5} - 5.8×10^{-7} per generation (Table 11); these frequencies were considered too high for mutagenesis, and a substitute strain (80550, A-layer negative) showed low frequencies of spontaneous mutants to kanamycin. However, *A. salmonicida* 80628 showed low frequencies of spontaneous kanamycin-resistance on many occasions. These results also indicate that *A. salmonicida* 80628 can readily acquire spontaneous resistance to kanamycin which could confuse the detection of Tn5 mutagenesis.

4.4 - Transposon mutagenesis of *A. salmonicida* 80628 using different vectors

Plasmids vectors other than pUW 964 were also used to generate mutants. Where plasmid vectors chosen were not mobilizable a plasmid helper was also used. These vectors are characterized by their sizes when compared to pUW 964; plasmids pLG 221, pGS 9, and pSUP 202-1 are constructed plasmids carrying Tn5. Plasmid pRK 2013 was used as helper and *E. coli* was the donor strain.

Matings were carried out by mixing fresh cultures of each *E. coli* donor strain with *A. salmonicida* 80628 and *E. coli* carrying pRK 2013 at ratios 1/1/1 for 24 h on filters; mixtures were plated on selection plates and incubated for 3 days at 22 °C. Selection plates were supplemented with Fur ($20 \mu\text{g ml}^{-1}$) and Km ($25 \mu\text{g ml}^{-1}$). After 3 days of incubation no colonies appeared on the selection plates. Both donor and recipient strains grew when plated separately on BHI agar plates only.

4.5 - Expression of transposon Tn5 and Tn7 in *A. salmonicida* Tn5 mutants

To check whether the "suicide" plasmid pUW964 was maintained in *A. salmonicida* recipients, expression of transposon Tn5 and Tn7 in *A. salmonicida* 80628 transconjugants was investigated by plating 190 Km^R mutants on BHI agar plates containing antibiotics to which resistance was expected from the transposon markers (Km, Str, Tm, and Sp). Results (Table 12) showed that less than 2 % of mutants lost their expression of Km resistance, and 36.8 % of mutants expressed resistance to streptomycin,

Table 11: Frequencies of spontaneous mutation of *A.salmonicida* to Kanamycin

<i>A. salmonicida</i> strains	Km ($\mu\text{g.ml}^{-1}$)	Frequencies of spontaneous Km ^R colonies
80628	100	4.6×10^{-6}
“	50	$< 0.4 \times 10^{-7}$
“	50	1.7×10^{-7}
“	50	3.8×10^{-5}
“	50	1.7×10^{-6}
“	25	3.2×10^{-6}
“	25	5.8×10^{-7}
“	25	1.3×10^{-6}
80550	100	$< 0.12 \times 10^{-7}$
“	50	3.6×10^{-8}
“	50	$< 1 \times 10^{-6}$
4700	25	$< 0.21 \times 10^{-8}$

Table 12: Antibiotic resistance of putative Tn5 mutants of *A. salmonicida* 80628. Mutants were screened for their resistance to Tn5 and Tn7 antibiotic markers. Numbers between brackets refer to the concentrations of antibiotics in $\mu\text{g/ml}$.

<u>Antibiotics</u>				<u>Frequency (%)*</u>
<u>Km (50)</u>	<u>Str (100)</u>	<u>Tm (100)</u>	<u>Sp (100)</u>	
R	-	-	-	32.6
R	R	-	-	36.8
R	R	R	-	8.9
R	R	R	R	12.1
R	R	-	R	3.1
R	-	R	R	0.5
R	-	-	R	1.0
R	-	R	-	3.15

* : of 190 mutants which were screened. R : resistant.

Km : kanamycin; Str: streptomycin; Tm: trimethoprim, Sp: spectinomycin.

representing the dominant proportion of mutants expressing transposon markers; resistance to both trimethoprim and spectinomycin was poorly expressed. However, 32.6 % of mutants did not express Tn7 markers; also, kanamycin and streptomycin resistance were expressed more frequently than other markers in *A. salmonicida*. Both Tn5 and Tn7 genes were expressed in different proportions indicating that their expressions are probably subjected to different regulation controls, consequently such results indicate that *A. salmonicida* 80628 mutants maintain the "suicide" plasmid vector pUW964.

4.6 - Characteristics of Km-resistant colonies

Colonies of Tn5 mutants which appeared on selection plates were very small (0.5 - 1.0 mm) with a smooth surface; their growth was very slow and occurred at 22 °C within 7 days of inoculation. No differences were observed among these mutants when cultivated on BHI agar plates only. On selection plates containing casein, mutants which were apparently protease-negative appeared as very small colonies with no clear zones of casein digestion after 24h of growth, but after 48h mutants showed a very small zone of digestion around the colonies. These colonies were picked and purified for further analysis.

4.7 - Plasmid analysis of *A. salmonicida* 80628 Tn5 mutants

To determine whether Tn5 had transferred to plasmids carried by *A. salmonicida*, the plasmid profiles of Tn5 transconjugants were analysed using the extraction procedure of Kado and Liu (1981). Results (Figure 8) showed different profiles for the plasmid contents of certain mutants, with loss of a specific plasmid band (4.4 kb) observed among mutants obtained from different matings. Also, the appearance of a new plasmid of approximately 15.4 kb was observed among mutants which lost the 4.4 kb plasmid band. This suggested that Tn5 (5.8 kb) had been transferred to an *A. salmonicida* plasmid in many mutants.

Figure 8: Agarose gel electrophoresis of plasmids of *A.salmonicida* Tn5 mutants.

Plasmid extraction was carried out using the method of Kado & Liu (1981). Numbers on the side refer to molecular weight (kb) of fragments of λ DNA digested with *Hind* III.

M : λ DNA *Hind* III digest.

1 : *A. salmonicida* 80628.

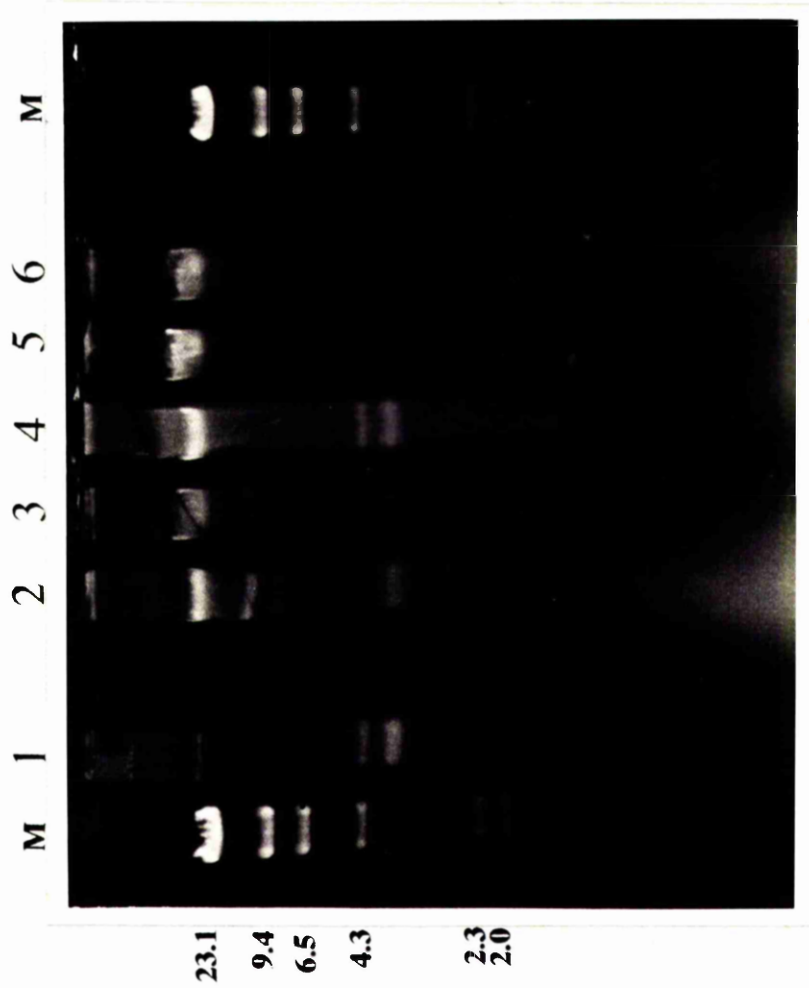
2 : mutant 80628-1/3.

3 : “ 80628-1/14.

4 : “ 80628-1/15.

5 : “ 80628-1/16.

6 : “ 80628-1/17.



4.8 - Caseinase and gelatinase activities of *A. salmonicida* 80628 Tn5 mutants

As a principal objective of this study was to obtain protease-negative mutants, this activity was measured in selection plates. Subsequently, protease assays were performed with azocasein using crude culture supernatants of mutants in BHI broth containing appropriate concentrations of kanamycin; colonies of mutants used were those which did not produce zones of clearance of casein digestion on selection plates after 24 h. The results (Figure 9) indicated that some mutants were weak producers of serine protease after 24h growth (about 50 % or less activity of the wild type), but this activity increased rapidly after 48h. These mutants still produced less activity after 48h of growth at 22 °C when compared to the parental strain 80628 and *A. salmonicida* 80550 (A⁻). Although mutants 80628-1, 80628-3, 80628-4, 80628-7, 80628-13, and 80628-16 did not digest casein on solid medium, they did produce some protease activity in liquid medium.

Detection of protease (gelatinase) in culture supernatants of mutants was achieved using overlaid BHI agar plates containing casein with 1 % (w/v) gelatin solution, and also using gelatin-PAGE analysis. All mutants tested produced gelatinase activity which was detected in both assays.

4.9 - Haemolytic activity of Tn5 mutants

Hemolytic activity of mutants was determined using horse blood agar plates and salmon blood "sandwich" agar plates. All mutants showed clear zones of haemolysis for both types of blood after 24 h incubation at 22 °C; mutants which were weak producers of caseinase also showed haemolysis. The zones of haemolysis were similar to those produced by the wild type strain 80628.

4.10 - SDS-PAGE of culture supernatants of *A. salmonicida* Tn5 mutants

When 24 h culture supernatants of mutants were subjected to SDS-PAGE analysis, the results showed (Figure 10) that of 18 mutants, 2 (Tracks 4 and 11) produced an extra protein band of approximately 50 kD. All mutants showed a band of Mr of 68 kD corresponding to the serine protease. No visible differences in protein

Figure 9 : Protease assay of *A. salmonicida* 80628 Tn5 mutants; the assay was performed on culture supernatants with azocasein as enzyme substrate. *A. salmonicida* 80550 was an A-layer negative strain; mutants 80628-1, 80628-3, 80628-4, and 80628-7 are weak protease exporters compared to their parental strain 80628.

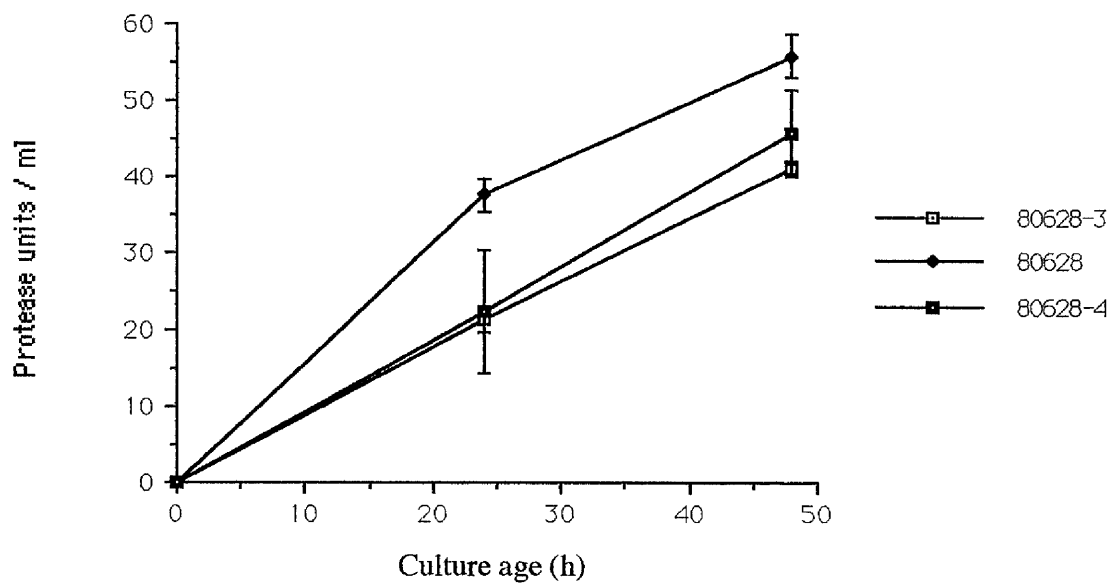


Figure 10: SDS-PAGE analysis of fresh culture supernatants of *A. salmonicida* Tn5 mutants grown in BHI broth. The gel was silver stained following electrophoresis. Numbers at the side refer to molecular weight in kD of protein markers.

M : SDS-6 protein markers (Sigma).

1 : mutant 80628-a/10.

2 : “ 80628-b/3.

3 : “ 80628-a/6

4 : “ 80628-1/3

5 : “ 80628-b/1

6 : “ 80628-b/4

7 : “ 80628-b/2

8 : “ 80628-1/2

9 : “ 80628-1/4

10: mutant 80628-1/1.

11: “ 80628-a/9.

12: “ 80628-a/1

13: “ 80628-a/4

14: “ 80628-a/8

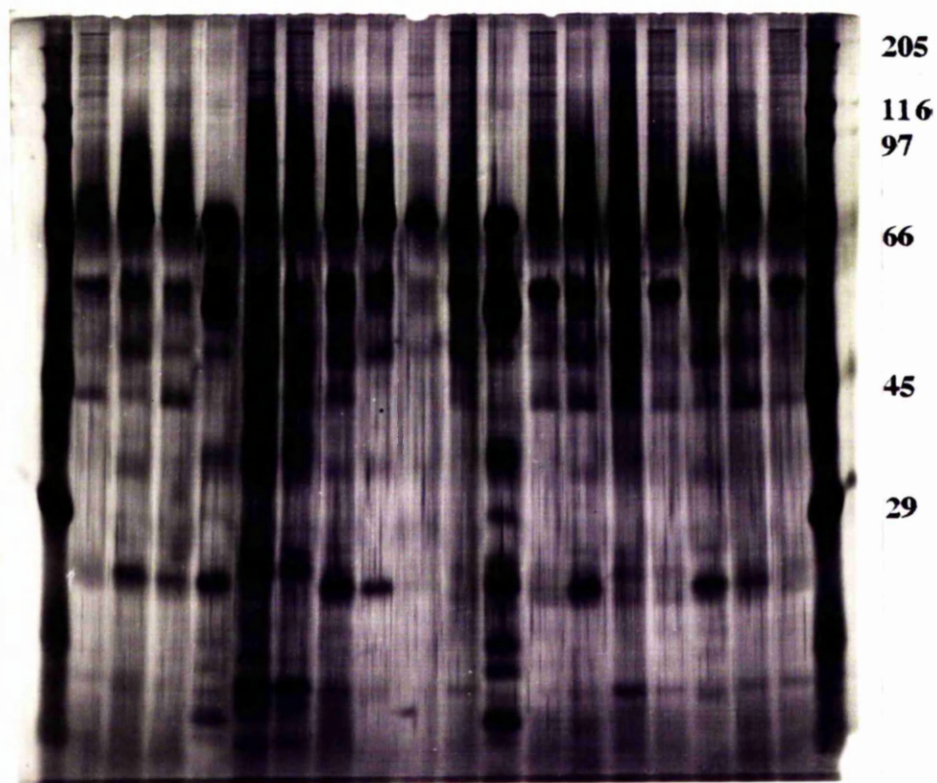
15: “ 80628-a/2

16: “ 80628-a/3

17: “ 80628-a/5

18: “ 80628-a/7

M 1 2 3 4 5 6 7 8 9 1 11 12 13 14 15 16 17 18 M



profiles of culture supernatants on SDS-PAGE of mutants were observed except for the two mutants mentioned above.

5 - Characterization of Tnp_{ho}A mutants

5.1 - Isolation of Tnp_{ho}A mutants

It was clear from the results obtained so far that there were several drawbacks to transposon mutagenesis using the recipient *A. salmonicida* strain 80628, namely the difficulties in using furazolidone as a selective agent, the tendency of Tn5 to insert preferentially into plasmids of strain 80628 and the frequency of spontaneous Km mutations. Therefore, an alternative plasmid delivery system carrying Tnp_{ho}A (a modified form of Tn5 which carries the structural gene for *E. coli* alkaline phosphatase without the signal sequence) was investigated. A fresh virulent isolate of *A. salmonicida*, 4700, was also used.

Preliminary observations of *A. salmonicida* Tnp_{ho}A mutants showed small colonies with different sizes and different surface characteristics. One major characteristic of these colonies was a blue colour due to strong alkaline phosphatase activity. This characteristic was not observed with *A. salmonicida* 4700 when grown on BHI agar plates supplemented with X-P (40 µg ml⁻¹). In liquid culture the same characteristic was observed, a uniform blue colour was visible after 36 h of culture of mutants at 22°C. Although *A. salmonicida* 4700 produced colonies with a dark blue centre, this strain did not produce this phenotype in liquid culture; all mutants which produced uniform blue colonies gave the same effect shown by mutants Pa3 and P7 in figure 11. Mutants selected from various matings were repeatedly cultivated on selection plates containing kanamycin, casein and X-P and those caseinase-negative were purified and stored in glycerol at -20 °C for further analysis.

Attempts to quantify the alkaline phosphatase activity of these mutants by measuring the amount of the blue color in the culture supernatants failed because it was cell-associated. Separating cells from culture supernatants by centrifugation (10,000 rpm

Figure 11: Characteristics of growth of *A. salmonicida* TnpHoA mutants on BHI broth supplemented with X-P.

- 1: *A. salmonicida* 4700 (parental strain).
- 2: mutant Pa3 (caseinase negative, Km^R).
- 3: mutant P7 (caseinase positive, Km^R).



1

2

3

for 10 min) showed that the blue color was all associated with the cell pellets and not in the culture supernatants. Furthermore, spectrophotometric analysis of these culture supernatants failed to detect any absorption over the wavelength range from 250 to 500 nm.

Interestingly, growth of mutants Pa3, Pa7, Pa10, and Pa18 on BHI agar plates showed differences in surface characteristics of the mutants when compared to their parental strain. These mutants produced rough colonies which were easily scraped from the surface of the agar plates; mutants Pa1 and Pa2 showed smooth colonies which, like the parent strain 4700, were difficult to remove from the plates when using a loop.

5.2 - Sensitivity of *A. salmonicida* TnphoA mutants to kanamycin

To determine the stability and expression of TnphoA, the antibiotic resistance of *A. salmonicida* TnphoA mutants was determined using BHI agar plates, containing a range of concentrations of Km, which were inoculated from overnight liquid cultures of mutants at a range of concentrations from 2×10^7 to 10^9 of bacteria. Results indicated that mutants were resistant to very high concentrations of Km ($550 \mu\text{g ml}^{-1}$) which corresponded to more than 10 times the concentration required to kill the same bacterial cell numbers of the parental strain (Table 13).

5.3 - Frequencies of TnphoA and spontaneous mutations of *A. salmonicida* strain 4700

Frequencies of TnphoA mutations obtained with recipient strain 4700 were close to those expected for Tn5 (Table 14), indicating a suitable system for mutagenesis which was much better than that previously used with recipient strain 80628. Moreover, the appearance of colonies spontaneously resistant to kanamycin was a very rare event in strain 4700.

Table 13 : Sensitivity of *A. salmonicida* TnpHoA mutants to kanamycin. Mutants were grown at 22 °C with shaking. Cell suspensions 0.1 ml, 2×10^7 to 10^9 bacteria, in the same medium were spread onto BHI agar plates containing kanamycin, and then incubated at 22 °C for 3 days.

Strain	Sensitivity to the following concentrations of kanamycin ($\mu\text{g/ml}$).							
	25	30	35	45	60	300	350	550
4700 (parent)	R	R	R	S	S	S	S	S
Pa1	R	R	R	R	R	R	R	R
Pa2	R	R	R	R	R	R	R	R
Pa3	R	R	R	R	R	R	R	R
Pa7	R	R	R	R	R	R	R	R
Pa10	R	R	R	R	R	R	R	R
Pa18	R	R	R	R	R	R	R	R

R = resistant; S= sensitive.

Table 14: Frequencies of Tnp_{hoA} mutagenesis of *A.salmonicida* 4700 and conditions of mating

Temperature of mating (°C)	Length of mating (h)	Nal ($\mu\text{g} \cdot \text{ml}^{-1}$)	Km ($\mu\text{g} \cdot \text{ml}^{-1}$)	Ratio <i>E.coli</i> / <i>A.salmonicida</i>	Frequencies of Km ^R colonies
22	24	30	25	1:1	7.6×10^{-5}
22	24	30	25	1:1	1.1×10^{-5}

5.4 - Characteristics of TnphoA mutants colonies on Coomassie Blue and on Congo Red agar plates

Coomassie blue and congo red agar plates supplemented with appropriate concentrations of kanamycin were used to detect loss of the A-layer of TnphoA mutants. On congo red agar plates all mutants produced pale red colonies indicative of A-layer expression; *A. salmonicida* 80550 (A-layer negative) was used as a negative control and showed growth of white colonies.

On coomassie blue agar plates, a distinct coloration of colonies was observed with mutants Pa3, Pa7 and Pa10 which produced pale blue colonies after 24 h of growth at 22 °C (Figure 12). The parental strain 4700, mutants Pa1, Pa2, and *A. salmonicida* FCC produced dark blue colonies when incubated at 22 °C for 24 h. Incubation of mutants Pa3, Pa7, and Pa10 for more than 3 days showed a change of colony colour to dark blue, in contrast to *A. salmonicida* 4700, mutants Pa1, Pa2, and *A. salmonicida* FCC which produced pale blue colonies when incubated for more than 3 days. It was concluded that mutants Pa3, Pa7 and Pa10 probably had altered surface characteristics involving the A-layer.

5.5 - Phage sensitivity of TnphoA mutants

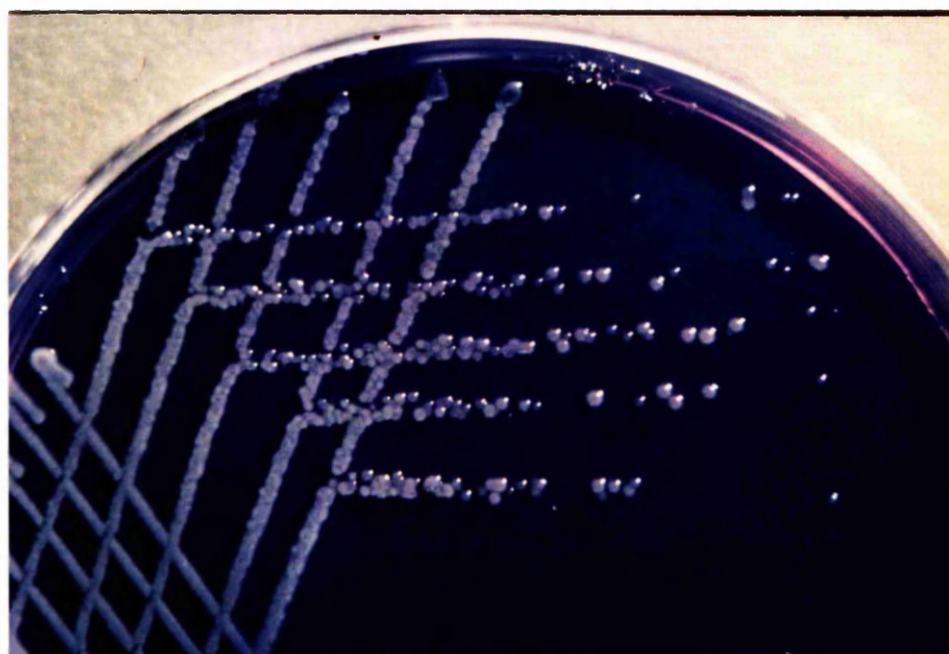
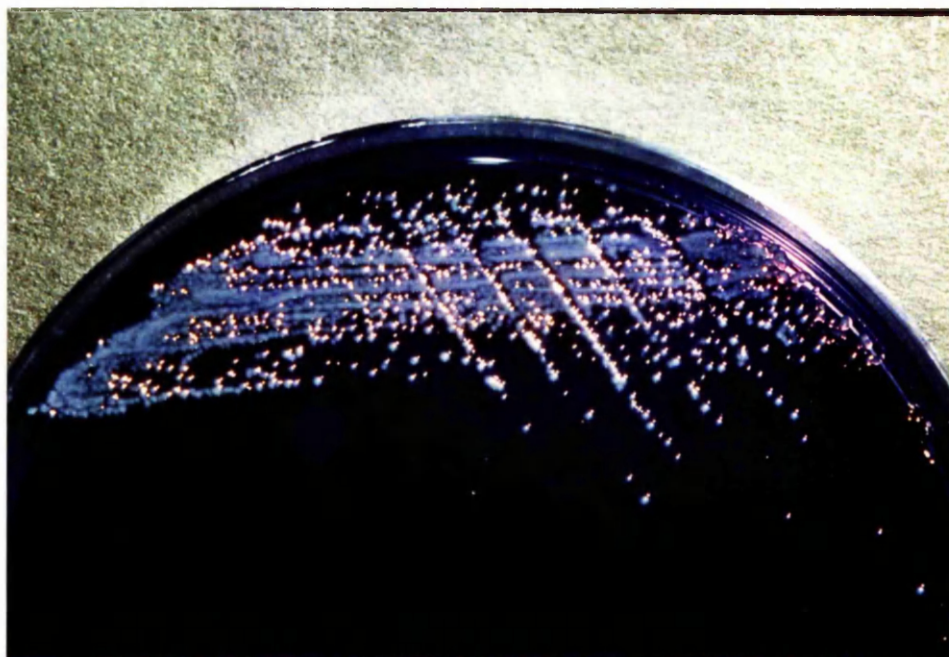
A range of *A. salmonicida* phages were available, three of which, ϕ_{1A} , ϕ_2 and ϕ_{13} , were known to bind to *A. salmonicida* LPS but not to the A-layer. *A. salmonicida* TnphoA mutants were tested for their sensitivity to these phages. Initial plaque assays with these phages and *A. salmonicida* 4700 resulted in a high virus titre indicating a high affinity of the wild type for these phages. Mixtures of an overnight cultures of mutants in BHI broth, with phages ϕ_{1A} , ϕ_2 and ϕ_{13} (at concentration of 10^8 /ml, 10^9 /ml and 10^9 /ml respectively), yielded no plaques for mutants Pa3, Pa7 and Pa10; mutants Pa1, Pa2 and Pa18 were fully sensitive to the phages and produced an uncountable number of plaques on agar plates.

Figure 12: Growth of *A. salmonicida* 4700 and mutant Pa3 on 0.1 % (w/v) Coomassie Blue-BHI agar plates.

A : *A. salmonicida* 4700.

B : mutant Pa3.

A



B

5.6 - Biochemical characteristics

5.6.1 - Caseinase activity of TnphoA mutants

The ability of *A. salmonicida* TnphoA mutants to produce and export caseinase was investigated during growth in both shaken and unshaken culture. High caseinase activity was observed during shaken growth of the wild type and mutant Pa18, with maximum activities produced during their stationary phases. Mutants Pa1, Pa2, Pa3, Pa7 and Pa10 produced activities varying from 5.4 % to 16 % compared to the parental strain 4700 (Figure 13).

During unshaken conditions of growth the activity of caseinase was considerably reduced for all mutants and the wild type (Figure 14). Mutants Pa1, Pa2, Pa3, Pa7 and Pa10 produced activities which were less than 10 % of that produced by the wild type.

5.6.2 - H-lysin activity of TnphoA mutants

The H-lysin activity was detected in static and shaken cultures respectively using blood agar plates and by haemolysin titration in microtitre plates. For static culture conditions mutants were simply inoculated onto BHI agar plates containing fresh washed horse blood; the H-lysin titration for shaken cultures was assayed at room temperature. The results showed reduced titres for mutants Pa1, Pa2, Pa3, Pa7 and Pa10 (Table 15), whereas no haemolysis occurred on horse blood plates (Figure 15).

5.6.3 - Titration of T-lysin activities of TnphoA mutants

The T-lysin was detected in 30 h culture supernatants of mutants using fresh salmon erythrocytes washed in sterile PBS. *A. salmonicida* 4700 and mutants Pa18 produced similar activities, but mutants Pa1, Pa2, Pa3, Pa7 and Pa10 produced less than 2 % of the activity of their parental strain 4700 (Table 16). The haemolytic effects of T-lysin was observed within a few minutes of mixing the culture supernatants of mutants with salmon blood suspension (Figure 16).

Figure 13 : Protease assay of *A. salmonicida* 4700 TnpHoA-generated mutants. The assay was performed on culture supernatants of shaken cultures. *A. salmonicida* 4700 represents the parental strain; mutants Pa1, Pa2, Pa3, Pa7 and Pa10 did not produce caseinase activity on casein-agar plates and they did not exhibit the 70 kD caseinase band on SDS-PAGE and western blotting; mutant Pa18 is a caseinase producer.

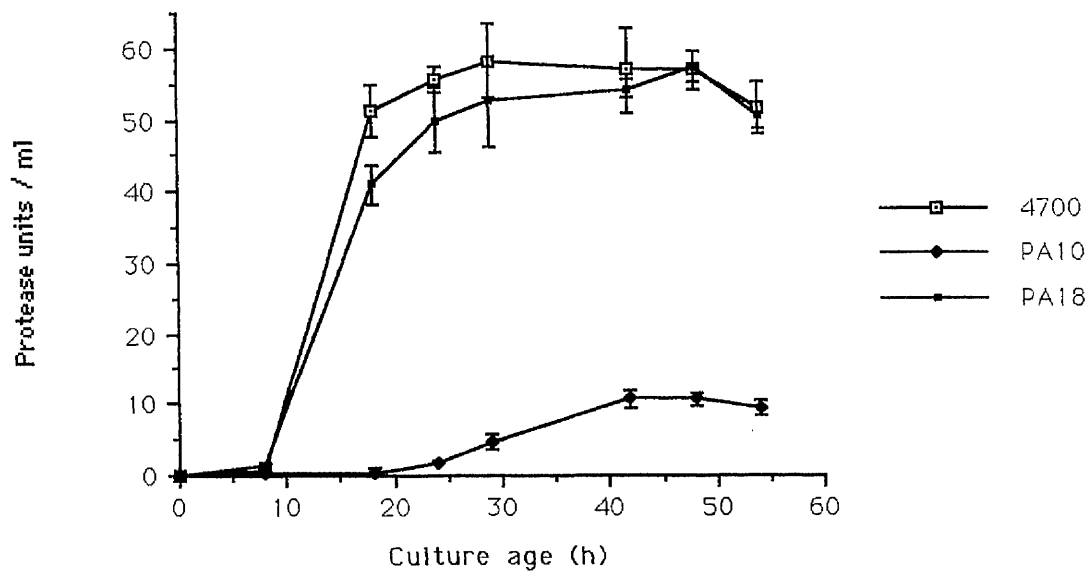
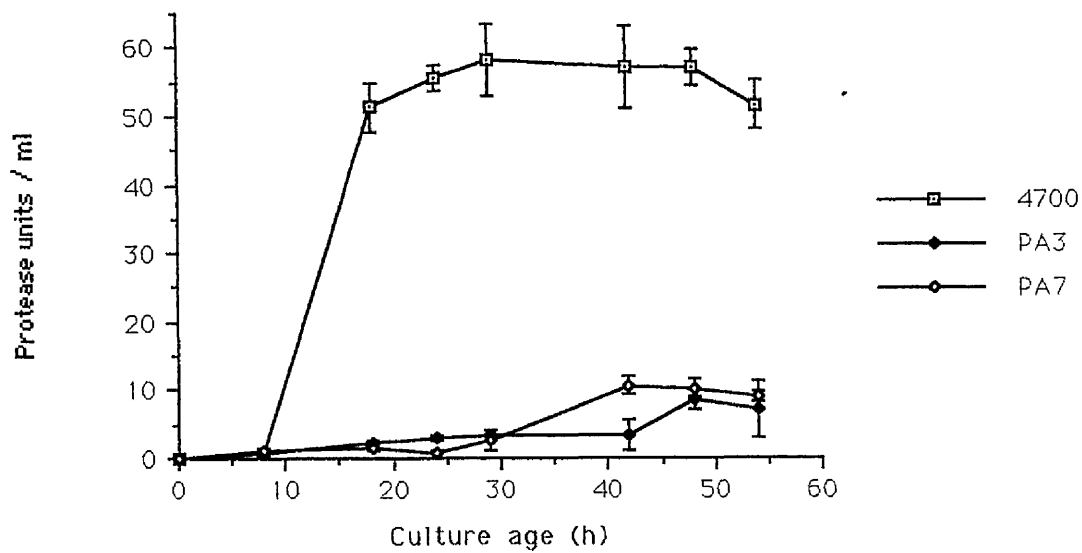
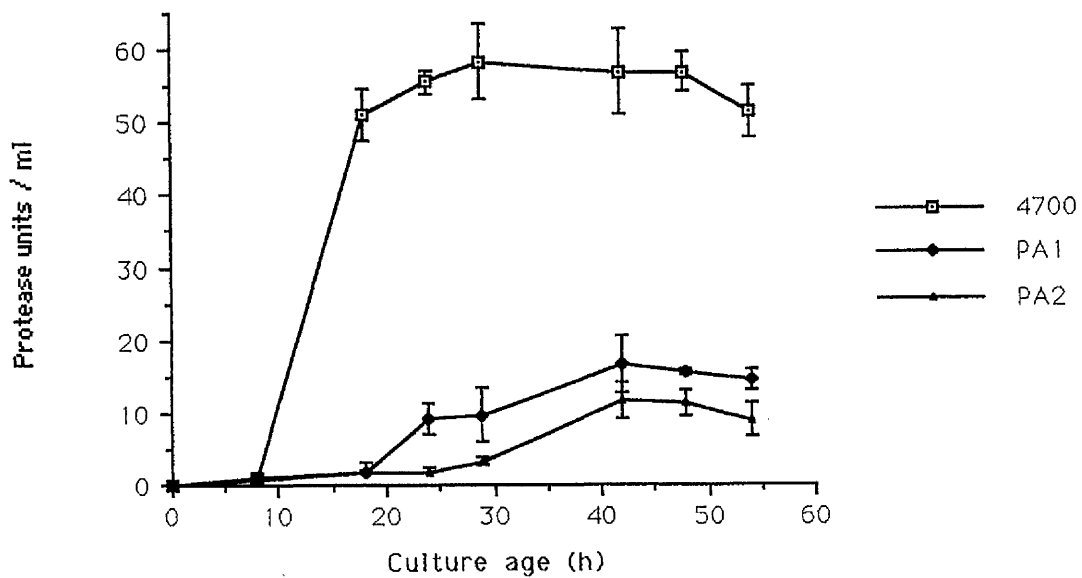


Figure 14: Protease activity of *A salmonicida* TnphoA mutants. The assay was performed using culture supernatants of static cultures of mutants in BHI broth as crude enzyme preparations, 1 % (w/v) azocasein (Sigma) was used as enzyme substrate, and one protease unit was defined as the amount of enzyme which produced an increase of 0.1 OD₄₄₀ units in 30 min at 37 °C.

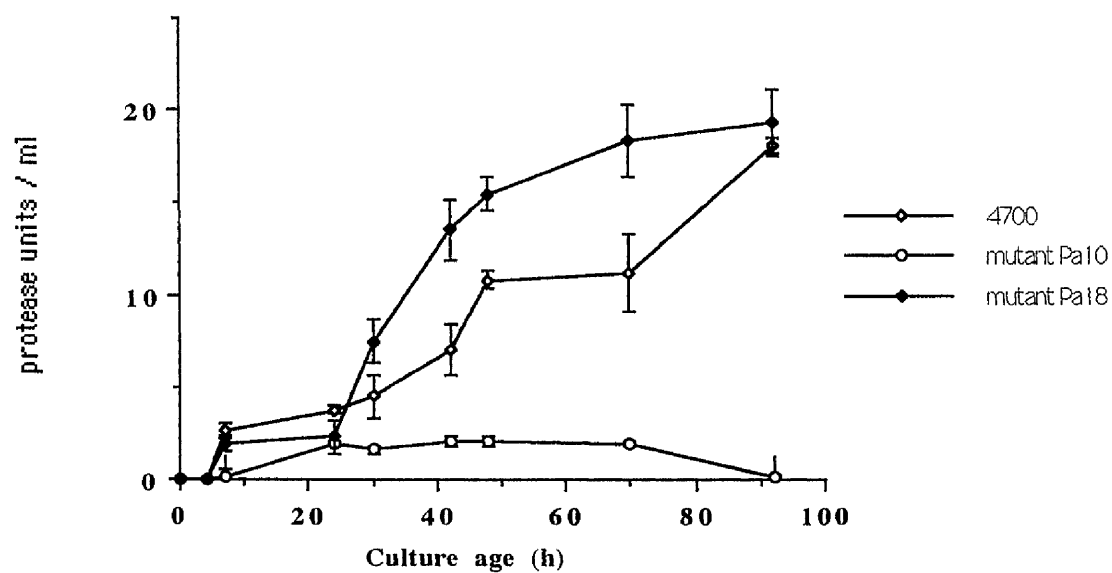
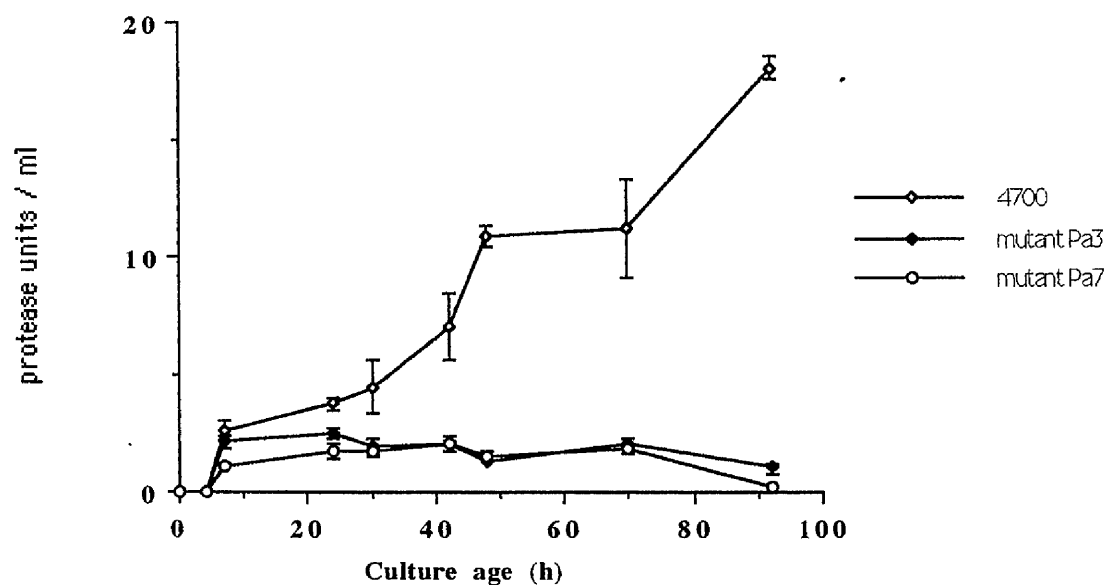
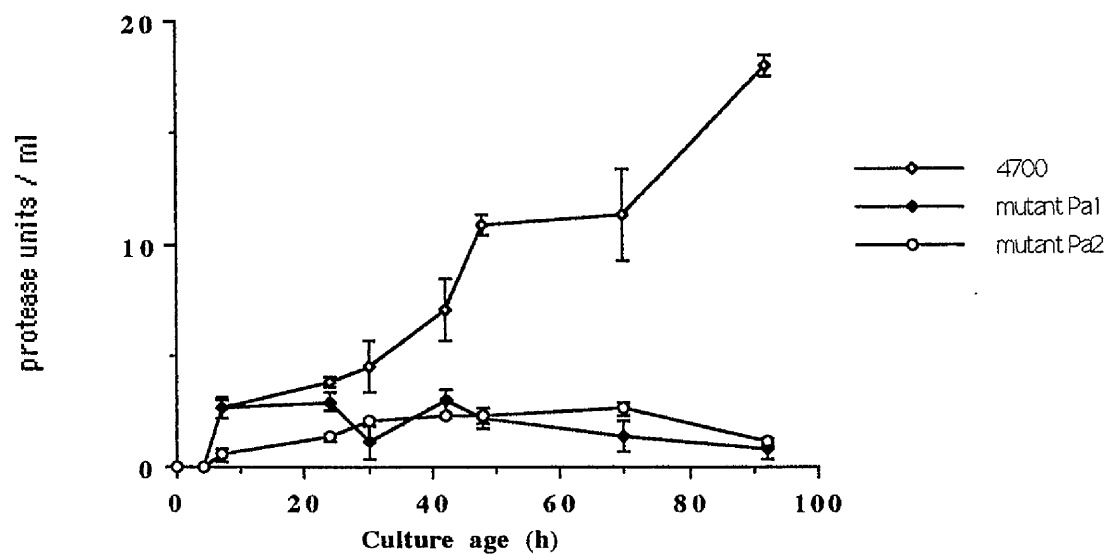


Table 15 : Haemolytic titres of fresh culture supernatants of *A. salmonicida* 4700

TnphoA mutants grown in shaken BHI broth cultures against horse blood cells. Titres were defined as the reciprocal of the last dilution showing clear and visible haemolysis.

Strain	Haemolytic activity (U ml ⁻¹)
<i>A.salmonicida</i> 4700 (wild type)	32
Mutant Pa 1	4
“ Pa 2	4
“ Pa 3	4
“ Pa 7	4
“ Pa 10	6
“ Pa 18	32

Figure 15: Haemolytic activity of *A. salmonicida* 4700 TnpHoA mutants on horse blood agar plates. Washed horse blood in sterile PBS was mixed with pre-cooled agar, and plates (7 %) were dried, U.V illuminated for 5 min and then immediately inoculated with mutants. Finally, plates were incubated at 22 °C for 30 h.

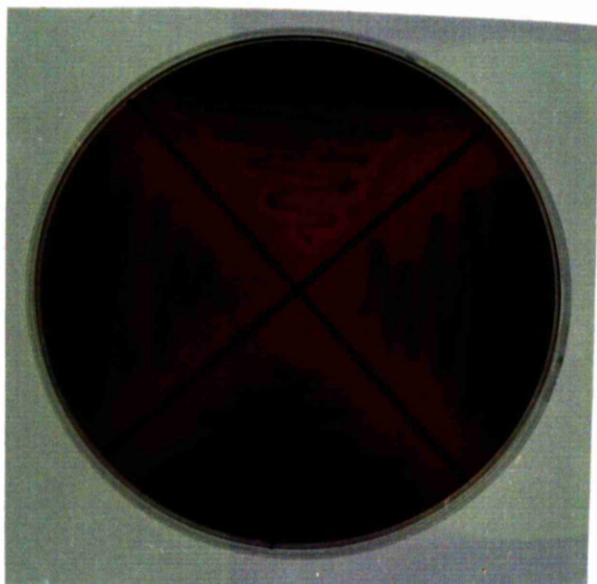
1 : *A. salmonicida* 4700 (parental strain).

2 : mutant Pa1.

3 : " Pa2.

4 : " Pa3.

1



4

2

3

Table 16 : Haemolytic titres of fresh culture supernatants of *A. salmonicida* 4700
TnphoA mutants against salmon blood. Haemolytic titres were expressed as the
reciprocal of the last dilution showing clear and visible haemolysis.

Strain	Haemolytic activity	
	T-lysin (units ml ⁻¹)	Activity (%) *
<i>A. salmonicida</i> 4700	1024	100
Mutant Pa 1	16	1.56
“ Pa 2	8	0.78
“ Pa 3	8	0.78
“ Pa 7	16	1.56
“ Pa 10	16	1.56
“ Pa 18	1024	100

* : Percentage of activity of mutants compared to their wild type *A. salmonicida* 4700

Figure 16: Haemolytic activity of *A. salmonicida* 4700 TnpHoA mutants on salmon blood. 30 h. culture supernatants of mutants were mixed with an equal volume of 1 % washed salmon blood in sterile PBS and incubated at 22 °C for 1 h. The haemolytic titres were expressed as the reciprocal of the last dilution which showed visible haemolysis.

1: washed salmon blood + BHI broth.

2: " + *A. salmonicida* 4700 (parental strain).

3: " + mutant Pa1.

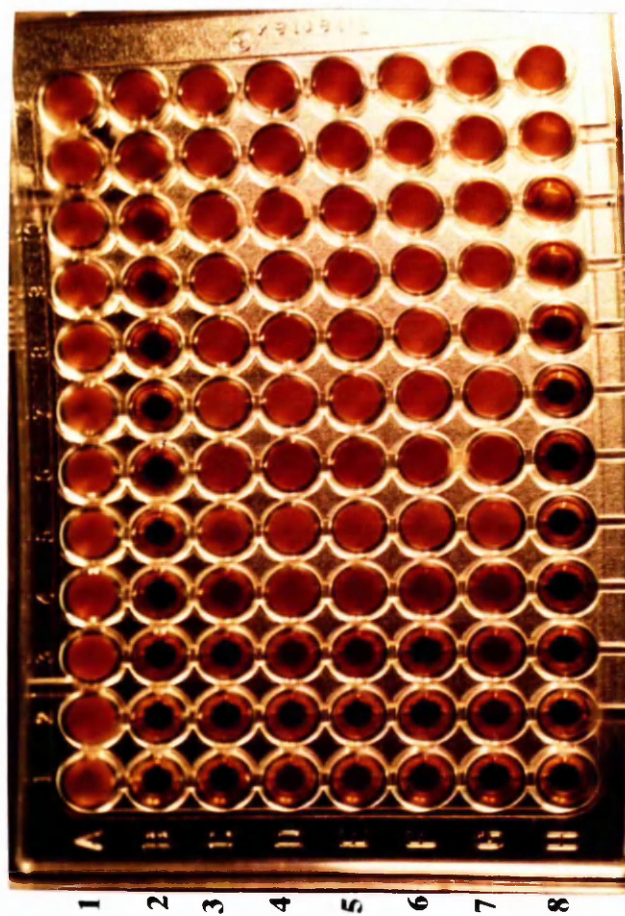
4: " + " Pa2.

5: " + " Pa3.

6: " + " Pa7.

7: " + " Pa10.

8: " + " Pa18.



5.6.4 - Phospholipase activity of TnpHoA mutants

Phospholipase activity of mutants was assayed using the conductimetric enzyme assay developed by Lawrence *et al.* (1971). The objective of the assay was to detect phospholipase activity, principally free GCAT activity, present in culture supernatants of mutants. The specificity, the sensitivity and the rapidity of the assay allowed one to perform the experiments on fresh culture supernatants without prior concentration and purification of the enzyme. However, presence of high concentrations of salt in the culture medium affected results in some cases. The usefulness of the assay permitted the differentiation of other phospholipases, such as phospholipase C (PLC), from phospholipase A₂-like (GCAT) activity present in mixtures by using stimulation and inhibition tests. Such tests were initially performed on commercial PLC, and results (Figure 17) showed that addition of CaCl₂ to the enzyme-substrate mixture increased PLC activity by approximately 3 times its initial activity. Moreover, addition of EDTA to the mixture inhibited virtually all activity. Thus, these results confirmed the characteristic of divalent-cation-dependence of bacterial phospholipases C studied so far. When similar tests were performed on concentrated culture supernatants of *A. salmonicida* strain 4700 and mutant Pa3 there was no inhibition by EDTA or stimulation by CaCl₂ of enzyme activities. This indicated that the phospholipase activity produced by strain 4700 was not PLC and that activity was probably due to free GCAT exported by *A. salmonicida*.

To check whether mutants produced phospholipase activities similar to that of the wild type strain 4700, the assay was performed on culture supernatants of various mutants and their parental strain. Mutants Pa1, Pa2, Pa3, Pa7 and Pa10 exhibited less activity when compared to their parental strain (Figure 18) suggesting that mutants lacking expression of the serine protease produced less activity than strain 4700; a protease-positive mutant (Pa18) produced a similar amount of activity to strain 4700. Phospholipase activity of *A. salmonicida* was readily detected after 30 h of growth; culture supernatants from exponential phase cultures of *A. salmonicida* 4700 showed a

Figure 17: Effect of EDTA and CaCl_2 on the activity of *A. salmonicida* glycerophospholipid:cholesterol acyltransferase and *Clostridium perfringens* phospholipase C. The activity was assayed by the conductimetric enzyme assay (Lawrence, 1971). The substrate (Dioctanoylphosphatidylcholine), final concentration $40 \mu\text{g/ml}$ in the buffer (triethanolamine), was equilibrated in the conductimetry cells prior to addition of enzymes.

A : $40 \mu\text{l}$ of fresh culture supernatants of *A. salmonicida* 4700, alone or in the presence of $20 \mu\text{l}$ of 100 mM of CaCl_2 or $20 \mu\text{l}$ of 100 mM of EDTA.

B : $5 \mu\text{l}$ (1 mg/ml) of phospholipase C solution was assayed in the presence of $20 \mu\text{l}$ (100 mM) of CaCl_2 or $20 \mu\text{l}$ (100 mM) of EDTA.

Arrow indicates addition of CaCl_2 solution.

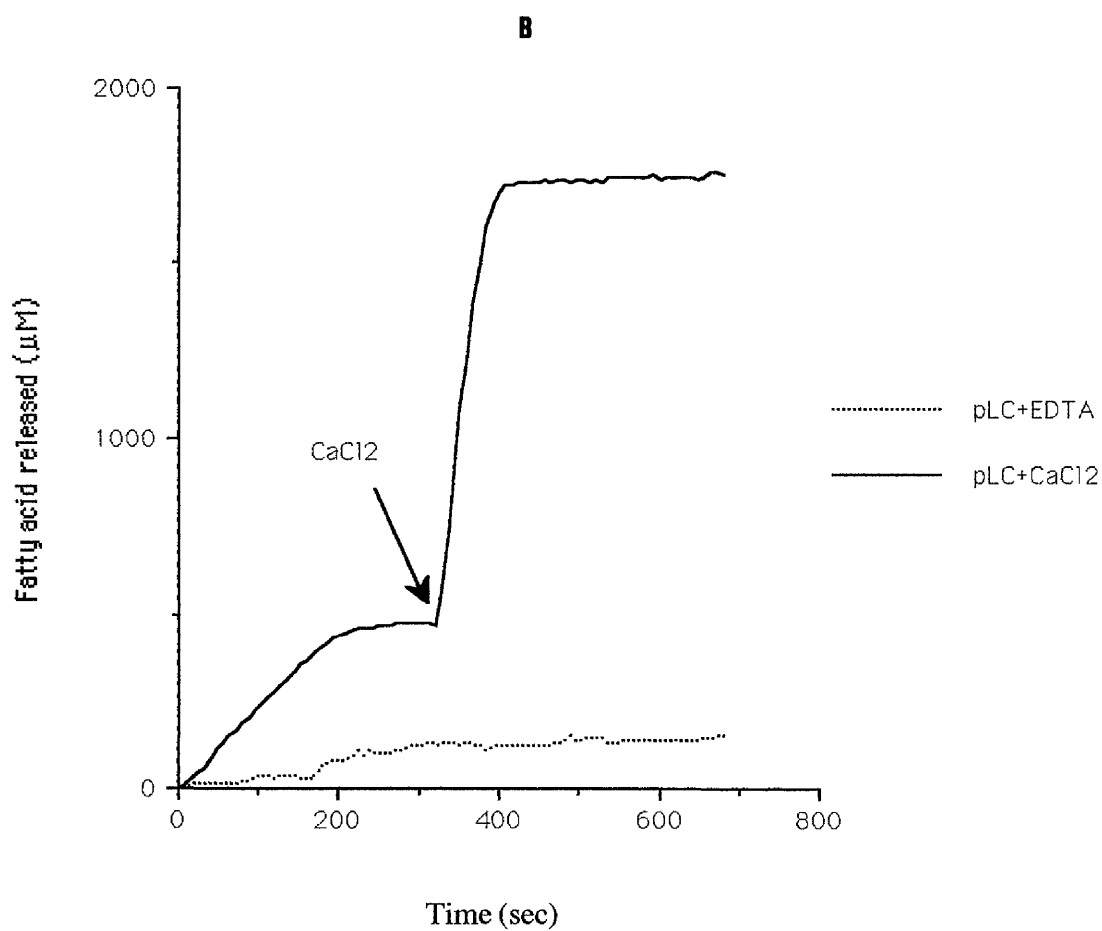
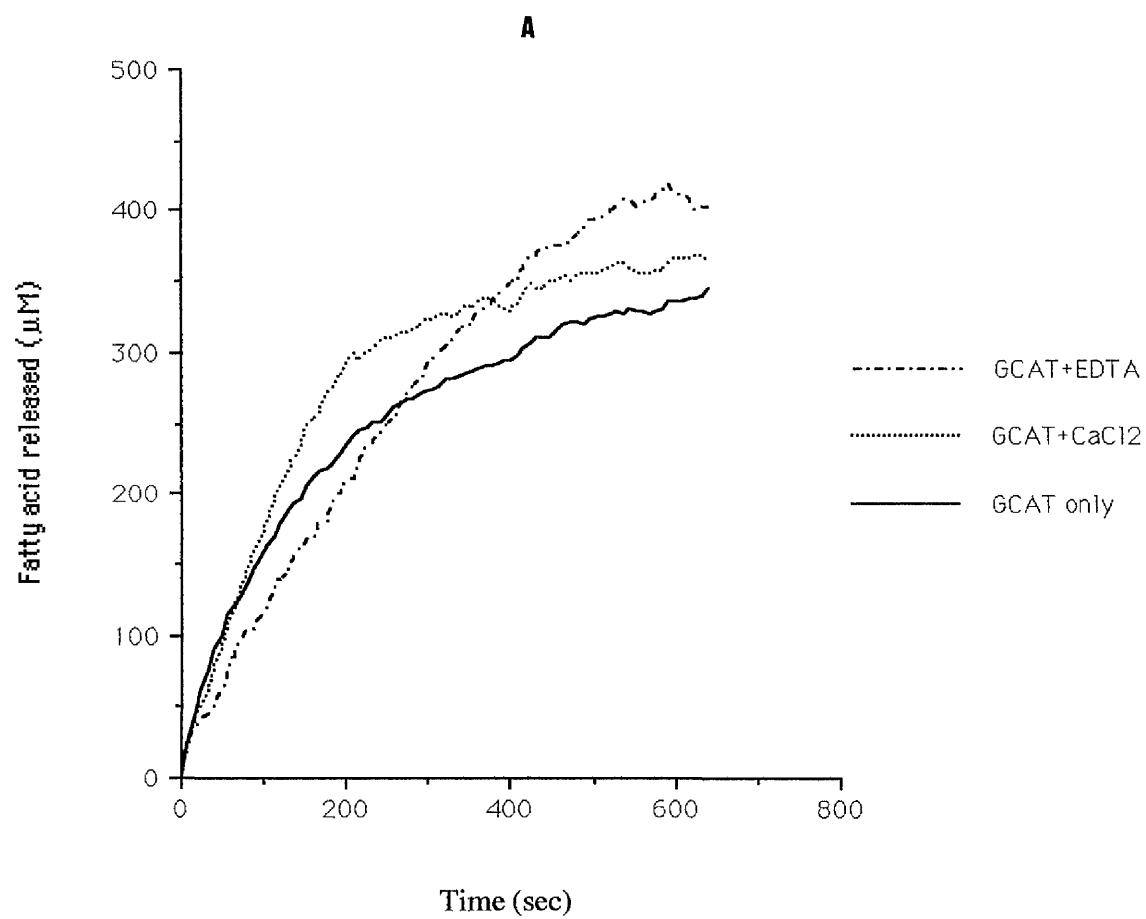
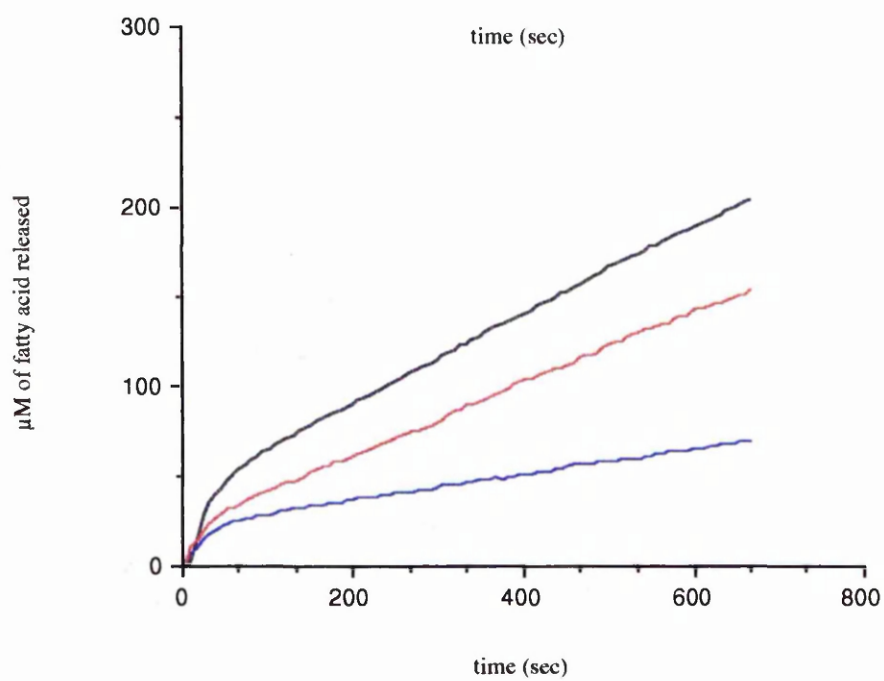
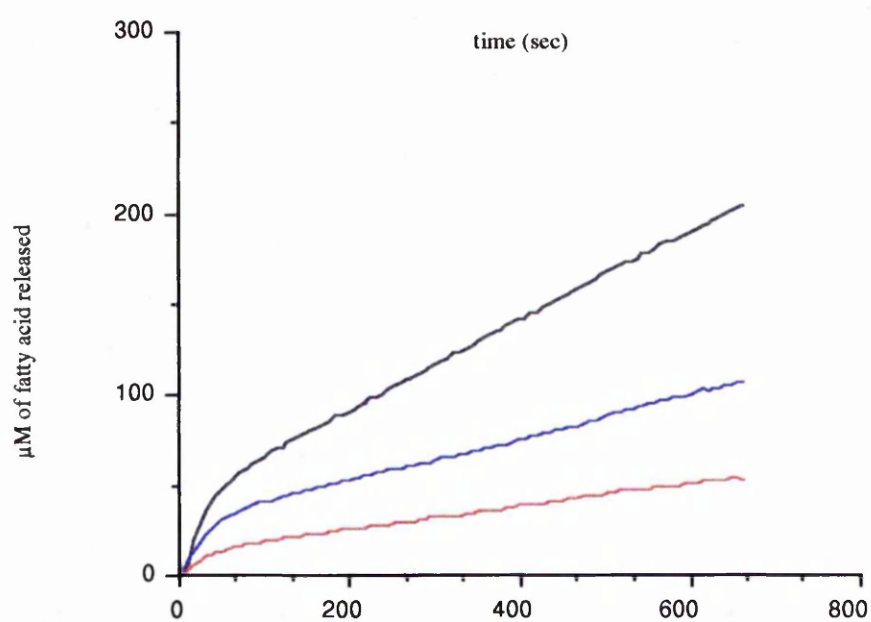
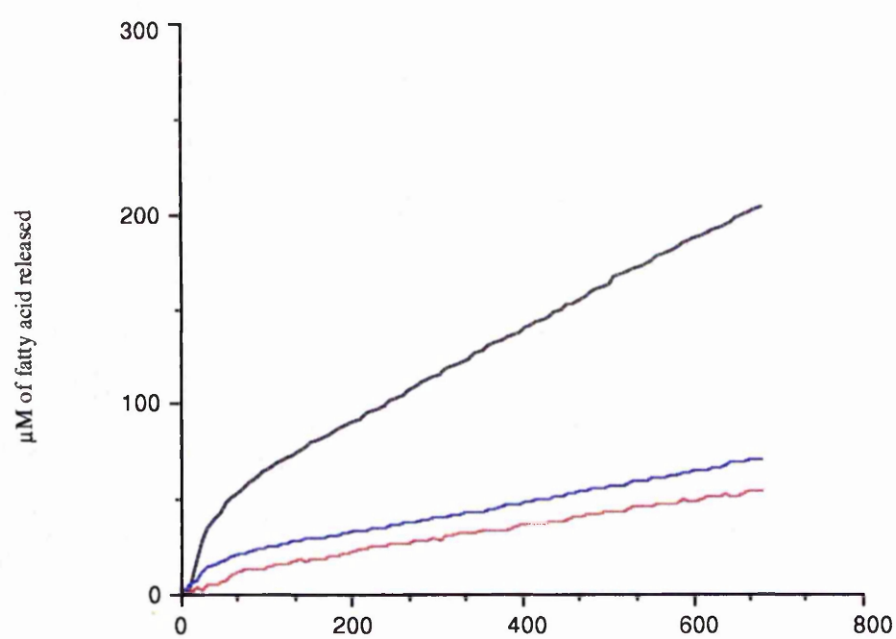


Figure 18: Phospholipase activity of *A. salmonicida* 4700 TnphoA-generated mutants. The activity of the GCAT was assayed on culture supernatants using the conductimetric enzyme assay of Lawrence *et al.* (1971); 40 μ l of crude enzyme preparation was added to 2 ml of buffer containing 2 μ l of substrate (40 mg.ml⁻¹ dioctanoylphosphatidylcholine).



small amount of activity but none was detected for the mutants listed above. All experiments showed that *A. salmonicida* 4700 released maximum phospholipase activity during the stationary phase.

5.7 - Protein analysis

5.7.1 - SDS-PAGE analysis of culture supernatants

Protein profiles of culture supernatants of *A. salmonicida* TnphoA were analyzed on 12.5 % polyacrylamide gels. Silver staining revealed a range of extracellular proteins (Figures 19, 20 and 21) in the wild type which were not found in mutants Pa5, Pa6, Pa7, Pa8, Pa9, Pa10, Pa11 and Pa12. These included a major band of approximately 70 kD which corresponded to the serine protease described earlier (see Section 1.3.2.2). This was absent from the profiles of these mutants, however, Km^R mutants Pa4 and Pa13 showed similar profiles to *A. salmonicida* 4700 (Figure 20). Mutants Pa3, Pa7, Pa10 and Pa18 displayed a particularly prominent band of approximately 50 kD which was considered to be the A-protein exported by these mutants. The expression of a 39.3 kD protein was observed in mutants which did not express the 70 kD serine protease but it was not observed in *A. salmonicida* 4700 or mutants Pa4 and Pa13.

5.7.2 - SDS-PAGE of whole cells of *A. salmonicida* TnphoA mutants

Bacterial cells of mutants grown in BHI broth at 22 °C for 24 h were treated with solubilizing buffer and then boiled for 5 min. Samples were analysed on 12.5 % SDS-PAGE and stained with coomassie blue. No significant differences in protein profiles were observed except for mutant Pa3 which showed a heavy band of approximately 50 kD (A-protein); the same protein band was observed in *A. salmonicida* FCC but not in *A. salmonicida* 4700 (Figure 22).

5.7.3 - SDS-PAGE analysis of outer membrane proteins of TnphoA mutants

Outer membrane proteins of mutants were extracted with sodium N-lauroylsarcosinate as described in Material and Method (see Section 3.18) were subjected to SDS-PAGE analysis. The molecular weights of the major proteins obtained varied

Figure 19: SDS-PAGE analysis of fresh culture supernatants of *A. salmonicida* TnphoA mutants. The 12.5 % gel was silver stained, and numbers at the side refer to molecular weight in kD of SDS-7 protein markers (Sigma).

M : SDS-7 protein markers (Sigma).

1 : *A.salmonicida* 4700 (parental strain).

2 : mutant P 7.

3 : " Pa1.

4 : " Pa2.

5 : " Pa3.

M 1 2 3 4 5

66

45

36

29

24

20.1

14.2

Figure 20: SDS-PAGE analysis of fresh culture supernatants of *A. salmonicida* TnpHoA mutants. 100 μ l of culture supernatants of mutants grown at 22° C for 30 h in BHI broth supplemented with kanamycin (35 μ g/ml, w/v) was mixed with the same volume of solubilizing buffer, boiled, and finally 35 μ l of mixtures were loaded onto a 12.5 % acrylamide gel. Numbers on the right refer to molecular weights in kD of protein markers (Sigma).

M : SDS-7 protein markers (Sigma).

1 : *A. salmonicida* 4700 (parental strain).

2 : mutant Pa4.

3 : " Pa5.

4 : " Pa6.

5 : " Pa7.

6 : " Pa8.

7 : " Pa9.

8 : " Pa10.

9 : " Pa11.

10: " Pa12.

11: " Pa13.

M 1 2 3 4 5 6 7 8 9 10 11 M

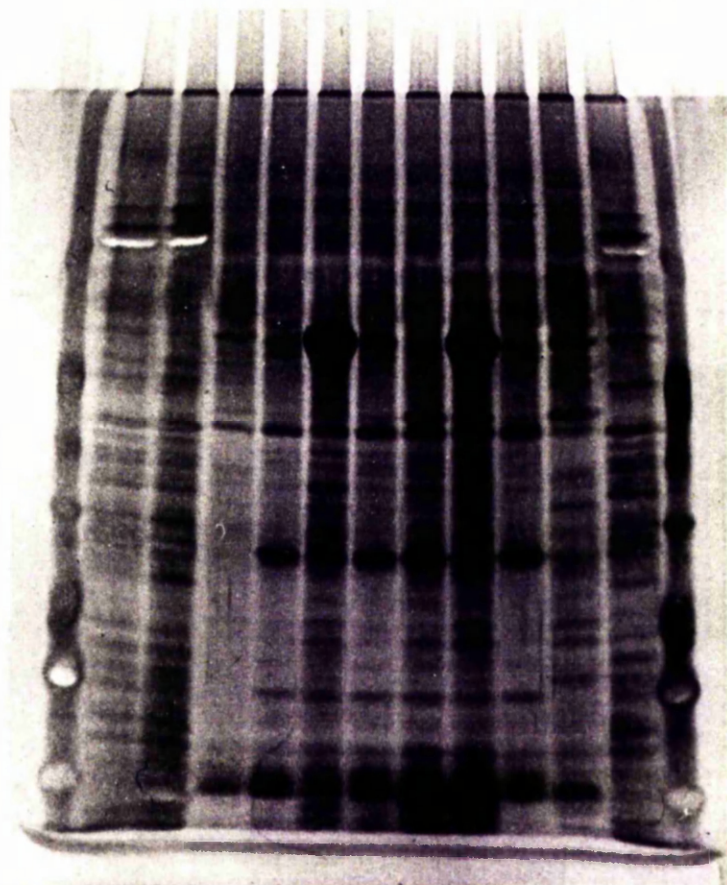


Figure 21: SDS-PAGE analysis of fresh culture supernatants of *A. salmonicida* TnpHoA mutants. The 12.5 % acrylamide gel was silver stained. Numbers on the right indicate molecular weight in kD of protein marker bands

M : SDS-7 protein markers (Sigma).

1 : *A.salmonicida* 4700 (parental strain).

2 : mutant Pa14.

3 : " Pa15.

4 : " Pa16.

5 : " Pa17.

6 : " Pa18.

7 : " Pa19.

8 : " Pa20.

9 : " Pa21.

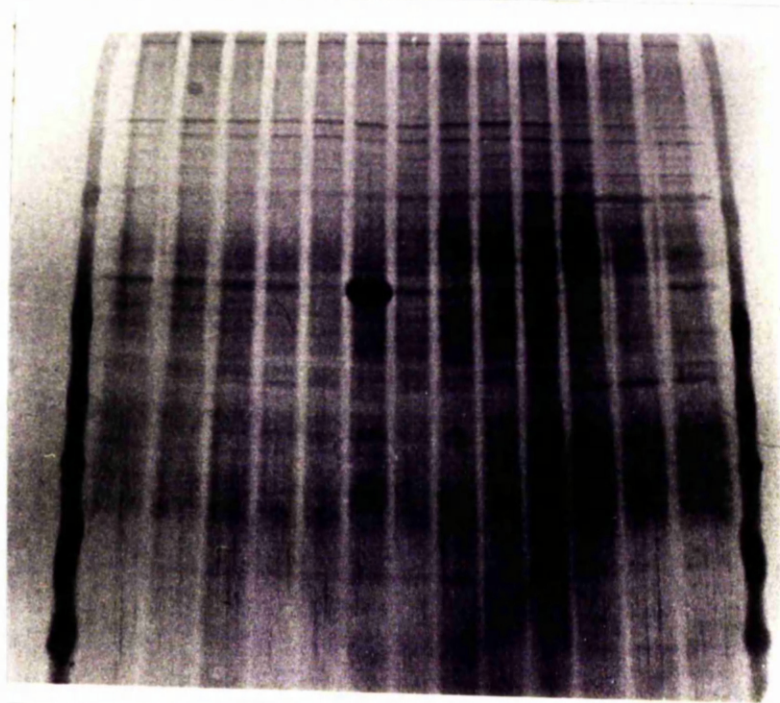
10: " Pa22.

11: " Pa23.

12: " Pa24.

13: " Pa25.

M 1 2 3 4 5 6 7 8 9 10 11 12 13 M



66

45

36

29

24

20.1

Figure 22: SDS-PAGE analysis of whole cells of *A. salmonicida* TriphoA mutants. Bacterial cell suspensions of mutants were mixed with solubilizing buffer, boiled for 5 min, then immediately loaded onto a 12.5 % acrylamide gel which was subsequently stained with Coomassie Blue. Numbers on the side refer to molecular weight in kD of protein marker bands.

M : SDS-6 standard protein markers (Sigma).

1 : mutant Pa10.

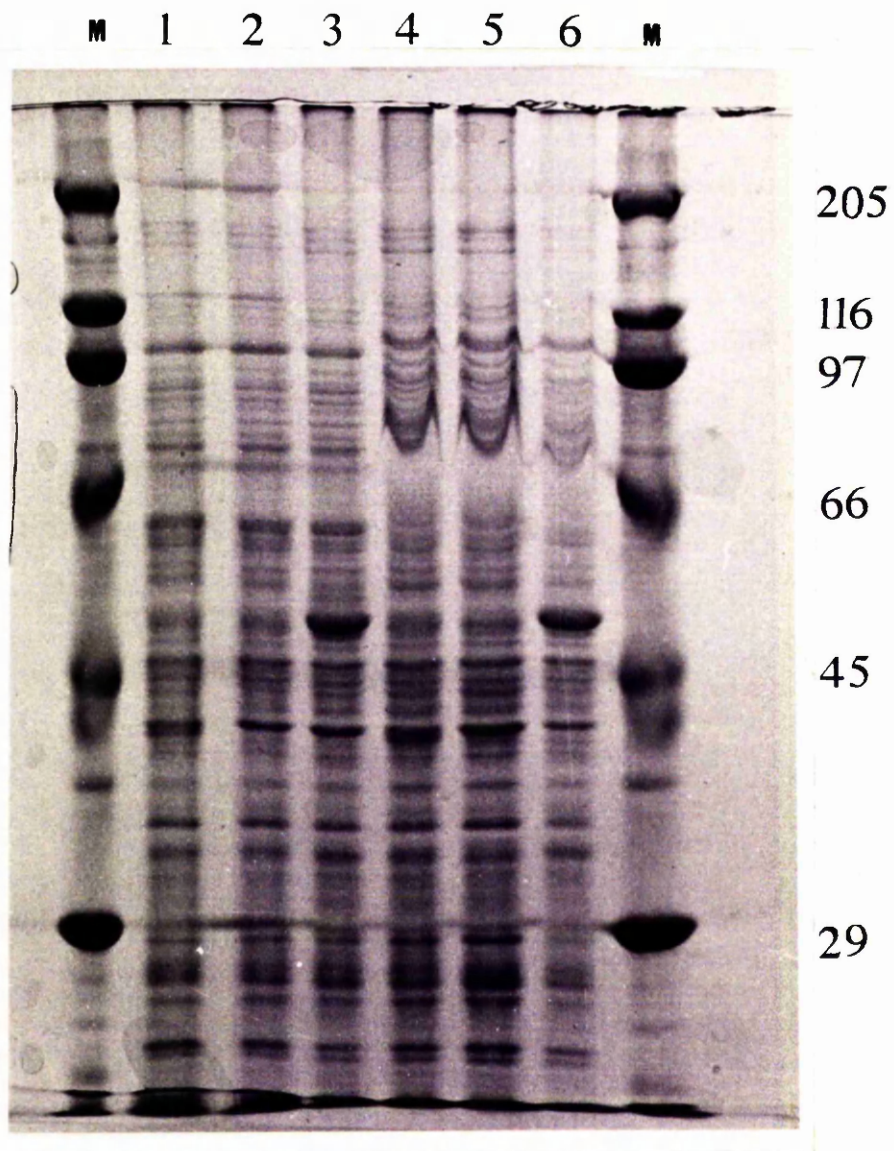
2 : mutant Pa7.

3 : " Pa3.

4 : " Pa1.

5 : *A. salmonicida* 4700 (parental strain).

6 : *A. salmonicida* FCC (virulent isolate possessing A-layer).



between approximately 20 kD and 80 kD. A distinguishing heavy band was observed in profiles of mutants Pa1 and Pa2; similar protein bands were observed in other mutants but at much lower intensity. The protein band of approximately 50 kD probably corresponded to the A-protein secreted by *A. salmonicida*. Another heavy band of approximately 39 kD was observed in all mutants studied including the parental strain 4700. Mutants Pa10 and Pa18 exhibited different protein profiles although they expressed the 39 kD protein band. They did not show the A-protein band and mutant Pa10 expressed a 46 kD protein band which was not observed in other profiles (Figure 23).

5.7.4 - Detection of gelatinase in culture supernatants of *A. salmonicida* TnphoA mutants

The gelatinase activity of TnphoA mutants was investigated using gelatin-PAGE. The assay was performed on 24 h culture supernatants of mutants loaded onto a gel containing 1 % (w/v) gelatin. Following electrophoresis and staining, clear zones of digestion of gelatinase were seen for all mutants used (Figure 24). Mutants Pa3, Pa7 and Pa10 showed faint digestion bands but more intense bands of gelatin digestion were produced by strain 4700 and mutant Pa18.

5.7.5 - Partial peptide mapping of the A-protein exported by mutants

To confirm that the 50 kD protein exported by various mutants was the A-protein, a partial peptide mapping was performed and the resulting bands were compared to purified A-protein. Bands from an SDS-PAGE gel were excised and digested with *N*-chlorosuccinimide (NCS). Digestion produced 4 major and 2 minor bands which were similar for all protein analysed (Figure 25) suggesting that mutants exported A-protein which was similar to that extracted from *A. salmonicida*, strain 80628.

5.7.6 - Immunological analysis of extracellular proteins of TnphoA mutants

Further evidence that the 50 kD protein exported by certain mutants was A-protein was obtained by immunoblotting. Exported proteins of the mutants were demonstrated immunologically after separation on 12.5 % polyacrylamide gels,

A-protein was purified from culture supernatants of *A. salmonicida* 80628 as described by Phipps *et al.* (1984).

Figure 23: SDS-PAGE analysis of outer membrane extracts of *A. salmonicida* TnpA mutants. The 12.5 % acrylamide gel was stained with Coomassie Blue. Numbers on the right refer to molecular weight in kD of protein marker bands.

M : SDS-7 protein markers (Sigma).

1 : *A. salmonicida* 4700 (parental strain).

2 : mutant Pa1

3 : " Pa2.

4 : " Pa3.

5 : " Pa7.

6 : " Pa10.

7 : " Pa18.

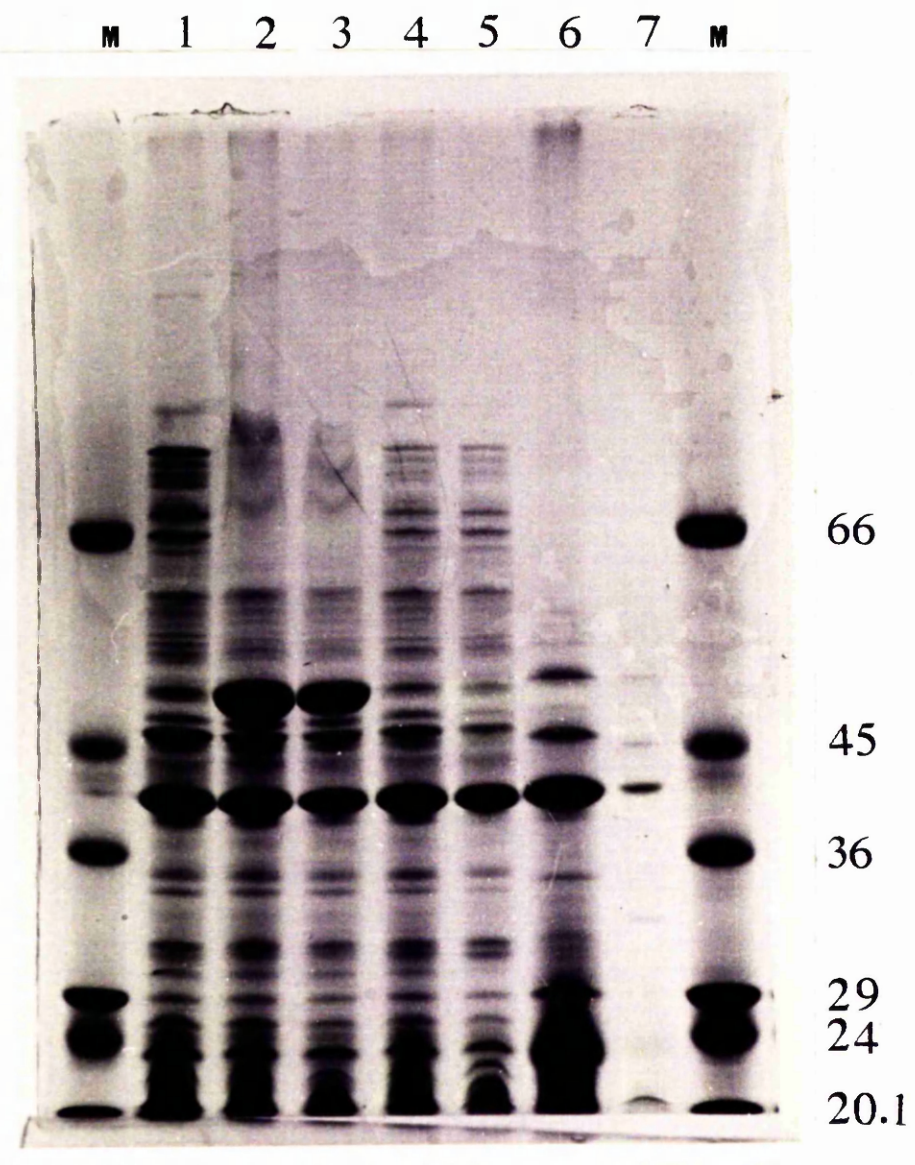


Figure 24 : Gelatin-PAGE analysis of *A. salmonicida* TnpHoA mutants. Fresh culture supernatants of mutants grown in BHI broth were loaded onto a 12.5 % polyacrylamide gel; following electrophoresis, the gel was stained with a solution of amido black (0.1 % w/v) as described in section (3.13.4).

1 : mutant Pa18.

2 : " Pa10.

3 : " Pa7.

4 : " Pa3.

5 : " Pa2.

6 : " Pa1.

7 : *A. salmonicida* 4700 (parental strain).

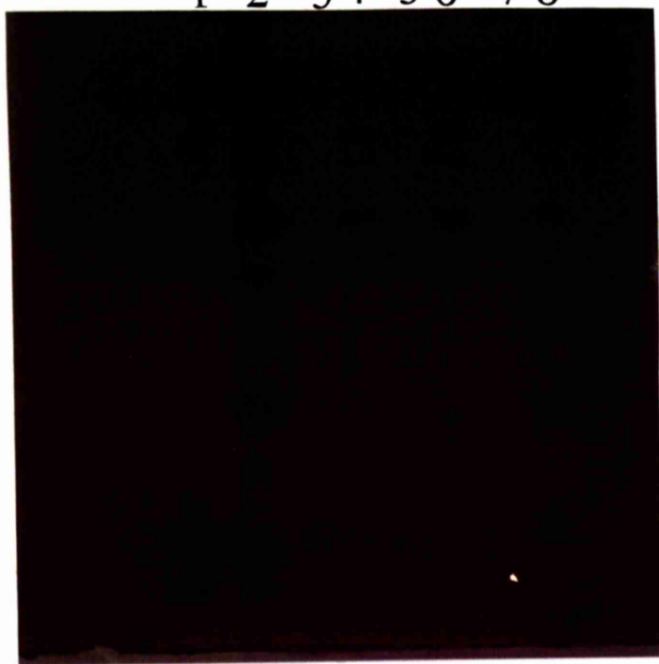
1 2 3 4 5 6 7



Figure 25 : SDS-PAGE analysis of A-protein digest of *A. salmonicida* TnpHoA mutants. Protein bands were previously excised from an SDS-PAGE gel treated with NCS using the method of Lischwe & Ochs (1982) and further analysed on a 12.5 % SDS-PAGE gel.

- 1 : untreated purified A-protein.
- 2 : NCS treated purified A-protein.
- 3 : untreated protein band of mutant Pa3.
- 4 : NCS treated " " Pa3.
- 5 : untreated " " Pa7.
- 6 : NCS treated " " Pa7.
- 7 : untreated " " Pa10.
- 8 : NCS treated " " Pa10.

1 2 34 56 78



electrotransfer to nitrocellulose paper and reaction with antiserum to *A. salmonicida* 80628 as described previously (Section 3.15.1).

24 h culture supernatants of mutants Pa1, Pa2, Pa3, Pa7, Pa10 and the parental strain *A. salmonicida* 4700 were used, with purified A-protein as a positive control. There was a strong reaction with a 70 kD protein exported by the parental strain (corresponding to the 70 kd serine protease); mutants Pa3, Pa7, and Pa10 reacted positively to one band corresponding to the A-protein, but did not react with the 70 kD serine protease (Figure 27). Mutants Pa1 and Pa2 did not show any reaction with the antiserum (Figure 26).

5.7.7 - Immuno-diffusion test of concentrated ECP's of *A. salmonicida* TnphoA mutants

The Ouchterlony immunodiffusion test was performed with rabbit anti-*A. salmonicida* 80628 serum and concentrated extracellular products of TnphoA mutants. Pa3 and Pa7 mutants (Figure 28) were chosen because they released A-protein into the culture supernatant. The results obtained with strain 4700 indicated that culture supernatant antigens other than A-protein, were recognised by the antiserum, among these antigens the 70 kD protease was a possible candidate since immunoblotting analysis of culture supernatants of the same mutants and strain 4700 revealed the 70 kD protease and the 50 kD A-protein.

5.8 - Southern blotting of chromosomal DNA of TnphoA mutants

Southern blotting of chromosomal DNA of TnphoA mutants was carried out to confirm insertion of transposon TnphoA. Initially, many attempts using plasmid pRT 733 as a probe for TnphoA failed, and difficulties were mainly encountered when extracting chromosomal DNA which seemed to be very unstable, probably due to nucleases. (Fig. 29) However, using plasmid pGLW 50 as a probe, TnphoA fragment was detected in all mutants except for the parent strain *A. salmonicida* 4700. Results (Figure 30) showed that the same band was obtained for all mutants and this may indicate that chromosomal

Figure 26: Immunoblot analysis of fresh culture supernatants of *A. salmonicida* TnphoA mutants using antiserum to *A. salmonicida* 80628 strain.

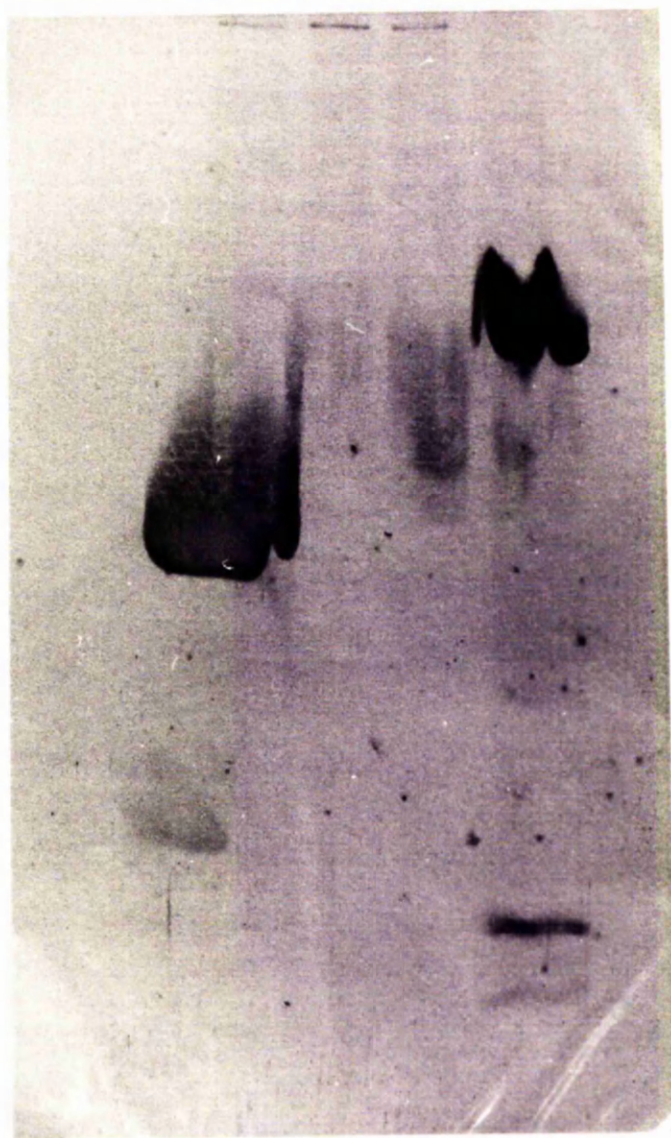
1: mutant Pa3.

2: “ Pa2.

3: “ Pa1.

4: *A. salmonicida* 4700 (parental strain).

1 2 3 4



— 70 kD

— 50 kD

Figure 27: Immunoblot analysis of fresh culture supernatants of *A. salmonicida*

TnphoA mutants using antiserum to *A. salmonicida* 80628 strain. Dilutions of purified A-protein were used for comparison.

1: 1/16 dilution of purified A-protein.

2: 1/8 “ “

3: 1/4 “ “

4: 1/2 “ “

5: neat (1 mg/ml).

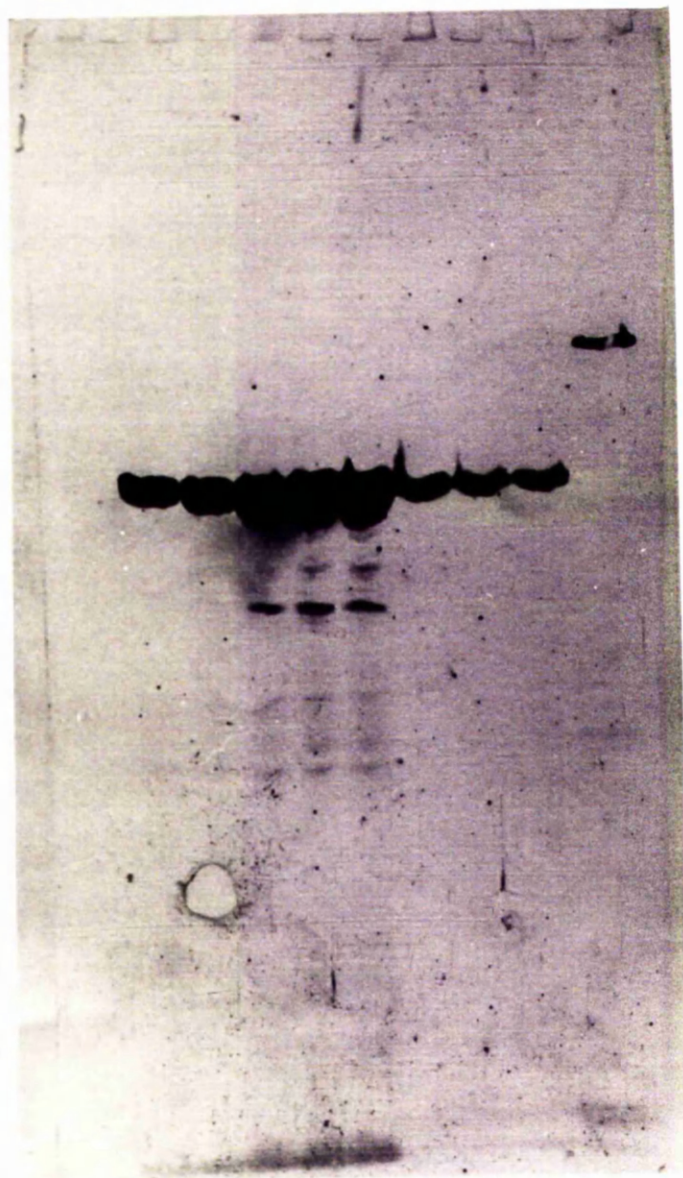
6: mutant Pa10.

7: “ Pa7.

8: “ Pa3.

9: *A. salmonicida* 4700 (parental strain).

1 2 3 4 5 6 7 8 9



— 70 KD

— 50 KD

Figure 28: Immunodiffusion test of fresh culture supernatants of *A. salmonicida* TnpA mutants using antiserum to *A. salmonicida* 80628.

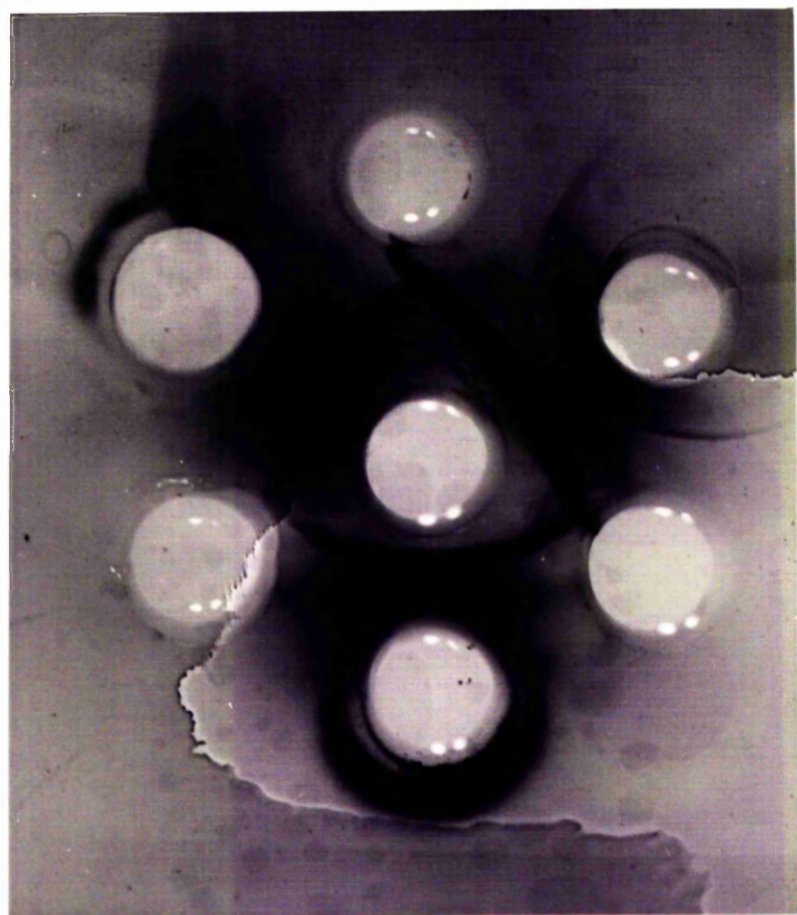
Central well : antiserum to *A. salmonicida* 80628.

1 : mutant Pa7.

2 : " Pa3.

3 : *A. salmonicida* 4700 (parental strain).

1



3

2

Figure 29: Agarose gel electrophoresis of restriction enzyme digest of plasmid pGLW50.

Plasmid pGLW50 was digested with *Xho* I and subjected to electrophoresis together with undigested plasmid pRT733. These plasmids were extracted using Quiagen plasmid Midi and Maxi preparations Kit (Diagen). The 4.8 kb Tn5 containing fragment was excised from gel and eluted using Gene Clean II kit (Hybaid) and then used as probe for TnphoA inserts. The sizes of λ *Hind* III digest are indicated on the left.

M : λ *Hind* III digest.

1 : plasmid pRT733.

2 : plasmid pGLW50 *Xho* I digest.

3 : undigested plasmid pGLW50.

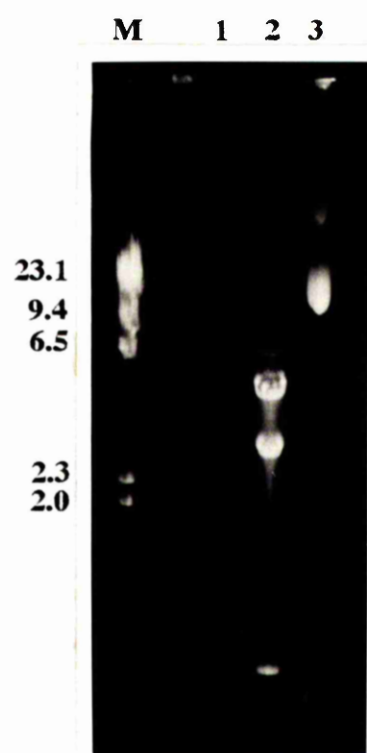


Figure 30: Southern blot analysis of *EcoRI*-digested chromosomal DNA of *A. salmonicida* TnphoA mutants probed with Tn5. Numbers refer to fragment sizes (kb) of 1 kb DNA ladder marker.

M : ^{32}P -1 kb DNA ladder marker (BRL).

1 : *A. salmonicida* 4700 (parental strain).

2 : mutant Pa1.

3 : " Pa2.

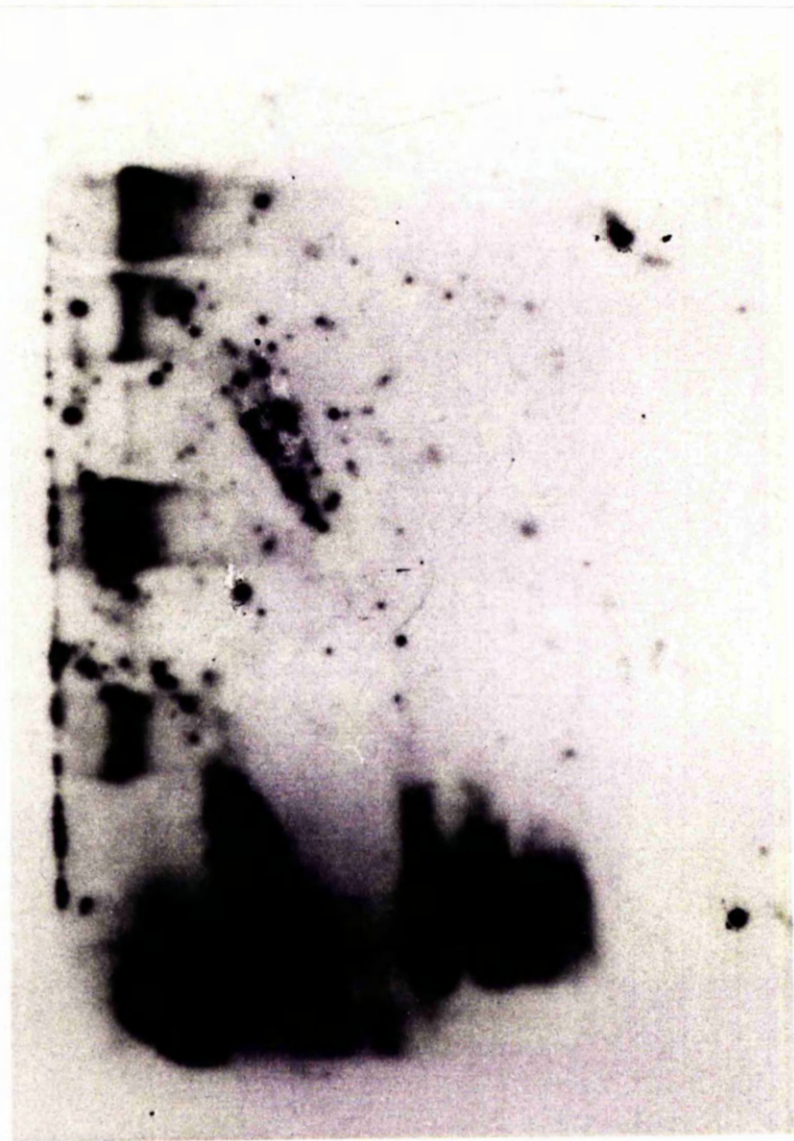
4 : " Pa3.

5 : " Pa7.

6 : " Pa10.

7 : " Pa18.

M 1 2 3 4 5 6 7



12.816 -

2.036 -

1.636 -

Figure 31: Agarose gel electrophoresis of plasmids of *A. salmonicida* TnpHoA mutants. Plasmid extraction was carried out using the method of Kado & Liu (1981). Numbers at the side refer to molecular weight (kb) of fragments of λ DNA digested with *Hind* III.

M : λ DNA *Hind* III marker.

1 : *A. salmonicida* 80628.

2 : *A. salmonicida* 4700.

3 : mutant P 9.

4 : " P 10.

5 : " P 11.

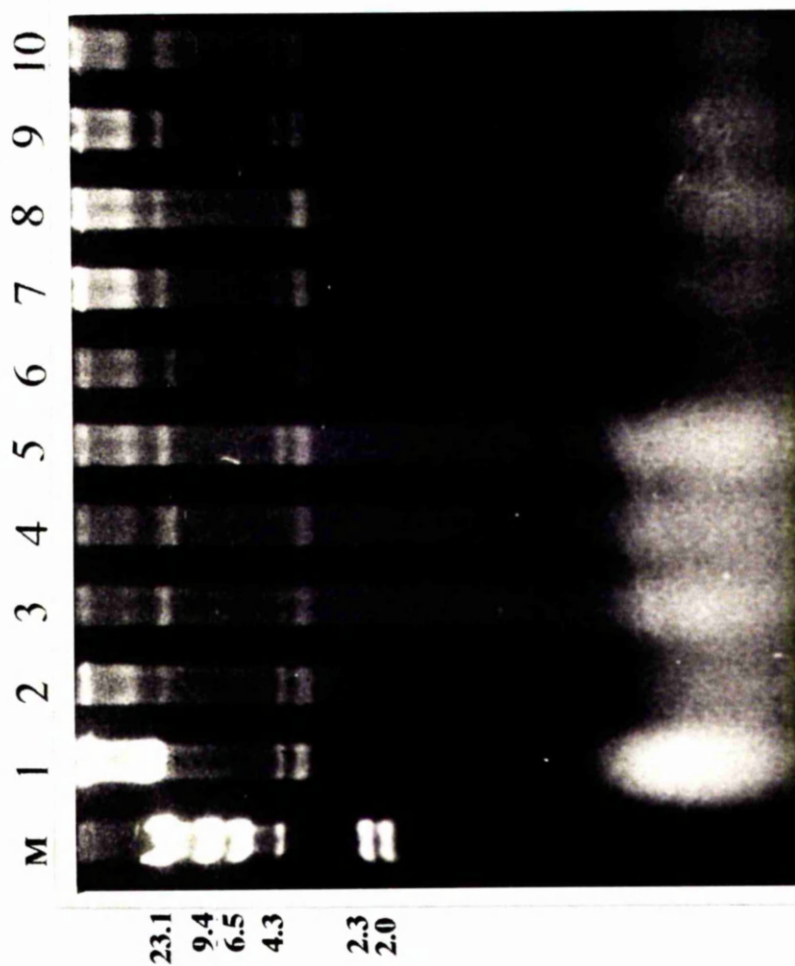
6 : " P 12.

7 : " P 13.

8 : " P 14.

9 : " P 15.

10: " P 16.



DNA of these mutants was partially digested by *EcoR* I. This was observed in several attempts.

5.9 - Analysis of plasmids of TnphoA mutants

Agarose gel analysis of plasmid profiles of TnphoA mutants showed plasmid bands similar to those observed with strain 80628 and the parental strain 4700. Two highly conserved bands of molecular weight ranging from 3.7 to 4.5 kb were observed in most mutants and a loss of plasmid band of 4.3 kb was also observed in certain mutants, but appearance of new bands was not observed (Figure 31).

Analysis of plasmid pGLW50 digest with *Xho* I showed five distinct fragments, a 4.8 kb Tn5 containing fragment was used as probe for TnphoA inserts. (Fig. 29)

5.10 - Electron microscope analysis of cell surface of TnphoA mutants

Cell surface characteristics of mutants were investigated by electron microscopy. Thin sections of cells observed with the microscope revealed major differences between strain 4700 and mutants Pa3, Pa7 and Pa10. Figures 32 and 33 showed a thinner outer surface layer in comparison to mutant Pa1. Also, mutant Pa3 showed a spiky cell surface when observed at 50 000 X magnification indicating that this mutant was not retaining materials on the outer cell surface, probably the A-protein. These characteristics were observed only with mutants Pa3, Pa7 and Pa10 which exported the A-protein into their culture supernatants; no differences were observed with mutants Pa1 and Pa2 when compared to their parental strain. This indicated that the A-layer could be multilayered. These results are consistent with others reported in this study which showed that mutants Pa3, Pa7 and Pa10 produced rough surface colonies and have reduced autoagglutinating capacity when compared to their parental strain.

5.11 - LPS analysis of TnphoA mutants

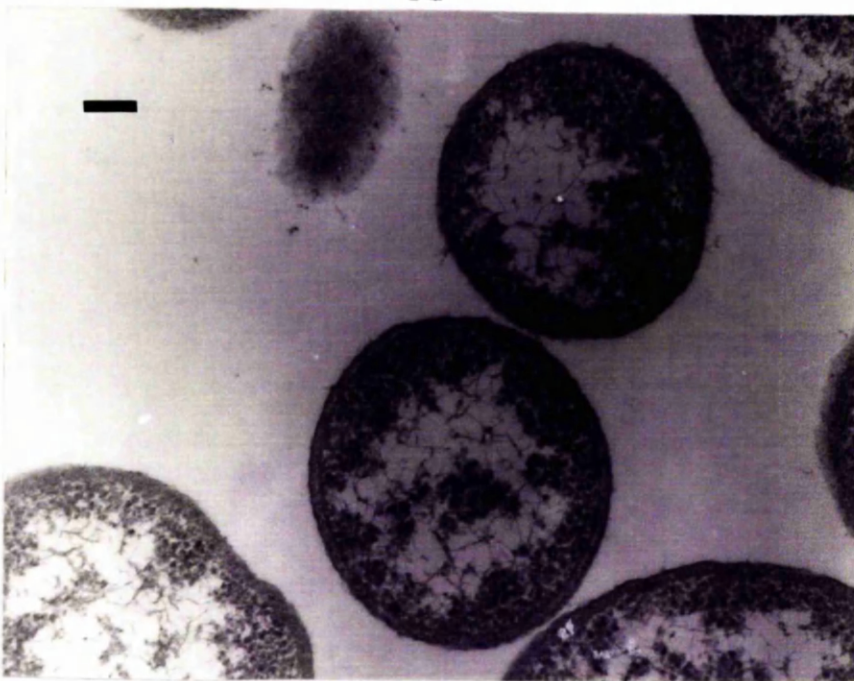
LPS profile analysis of TnphoA mutants (Figure 34) revealed the presence of smooth and high molecular weight LPS in mutants which did not export the A-protein,

Figure 32: Electron micrographs of *A. salmonicida* TnphoA mutants; bar represents 0.25 μm ; magnification 50,000 x.

A: mutant Pa1.

B: “ Pa3.

A



B

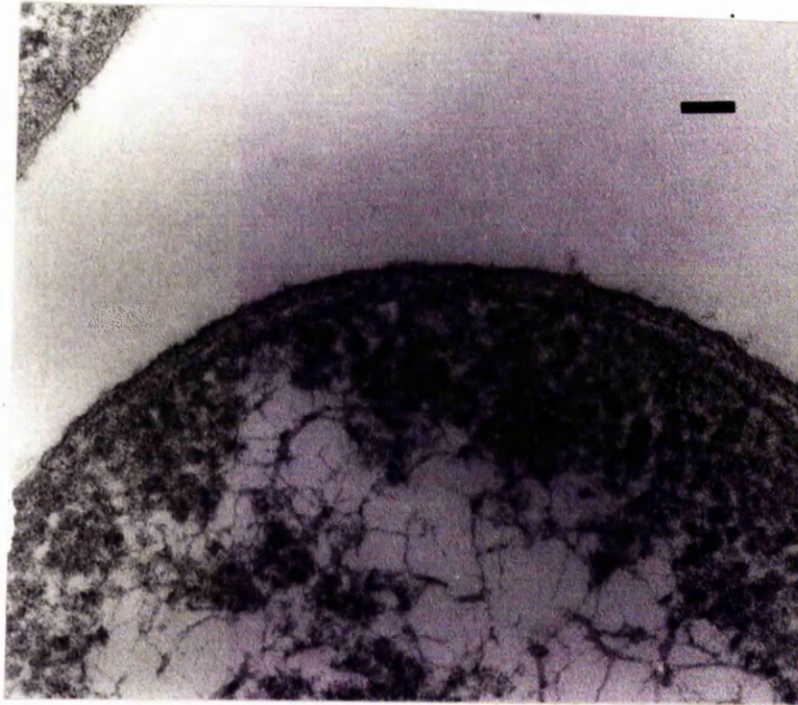


Figure 33: Electron micrographs of *A. salmonicida* TnphoA mutants; bar represents 0.09 μm ; magnification 85,000 x.

A: mutant Pa1.

B: “ Pa3.

A



B

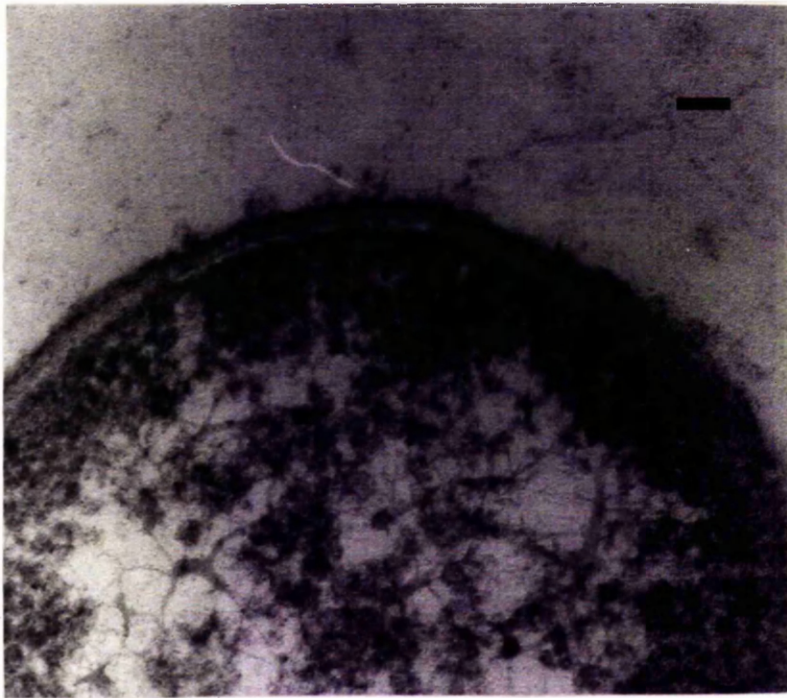


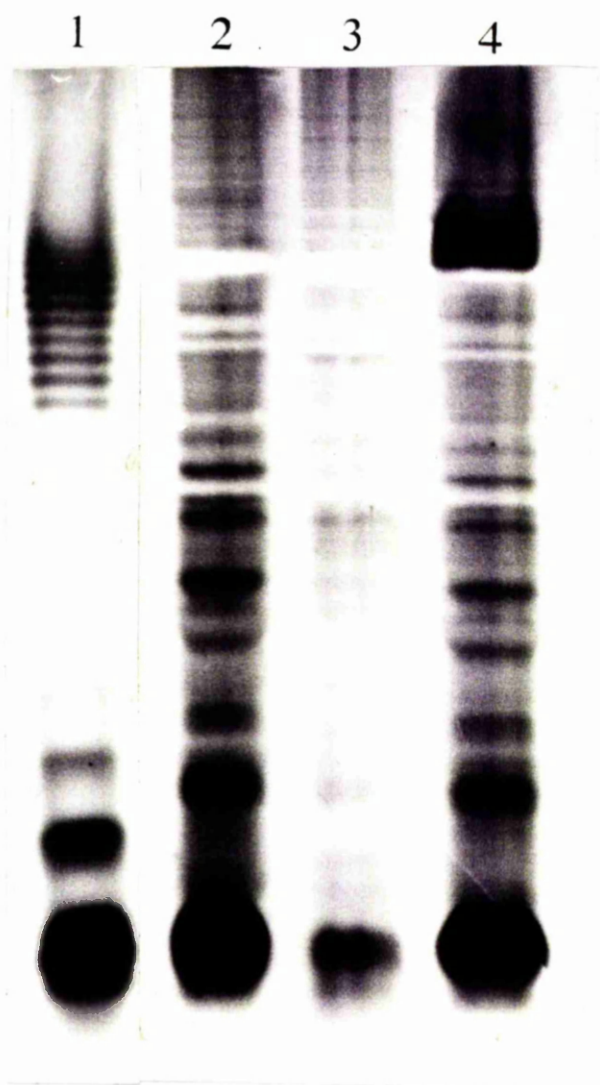
Figure 34: SDS-PAGE analysis of LPS of *A. salmonicida* . LPS was extracted from the TnphoA mutants using the method of Westphal & Jann (1965).

1 : standard smooth LPS marker of *E. coli* serotype 0111:B4 (Sigma).

2 : LPS of mutant Pa7.

3 : " " Pa3.

4 : " " Pa1.



but loss of these specific LPS was observed in mutants Pa3, Pa7 and Pa10 (not shown in Figure 34). No differences in rough and low molecular weight LPS were observed between mutants analysed.

Discussion

6.1 - Transposon mutagenesis of *A. salmonicida* 80628

Initially, studies were focused on finding a suitable transposon delivery system into *A. salmonicida*; such a system must yield transfer at high frequencies and not be maintained within the host. The "suicide" plasmid pUW964 has been used extensively for generating mutants of *Bordetella pertussis* lacking expression of virulence genes (Weiss *et al.*, 1983; Ward.M, 1990) and it was also used in mutagenesis studies with several *Pseudomonas* species (Boulnois *et al.*, 1985; Sokol, 1985; Cuppels, 1986). Reports on mutagenesis of *B. pertussis* using "suicide" plasmid pUW964 indicated a positive transfer between *E. coli* and *B. pertussis* (Weiss *et al.*, 1983). while Ward (1990) and Arain (1991) reported unsuccessful results. In this study plasmid pUW964 was used for mutagenesis of *A. salmonicida* 80628, and transfer of the plasmid to the host did occur. Evidence for transfer was obtained by analysis of drug-resistance acquisition of transconjugants as a result of expression of both Tn5 and Tn7 transposons carrying antibiotic resistance markers, and by the frequencies of appearance of kanamycin-resistant mutants. Frequencies of kanamycin-resistant mutations varied widely (2.4×10^{-2} to 2×10^{-8}); these frequencies were considered too high when compared to frequencies of spontaneous kanamycin-resistance of *A. salmonicida* 80628 (3.8×10^{-5} to $< 4 \times 10^{-8}$) obtained in this study, which suggests that spontaneous resistance to kanamycin is not a rare event in *A. salmonicida* 80628. The appearance of spontaneous mutants at such frequencies could be problematic in Tn5 mutagenesis, and could contribute to the variation of frequencies of Tn5 transconjugants obtained. Insertion of Tn5 into the chromosomes, plasmids and temperate phages of gram-negative bacteria was reported to be at high frequencies (10^{-2} - 10^{-5} per generation) depending on the host and target (Berg & Berg, 1983). Results obtained here with *A. salmonicida* 80628 suggested that this strain is not a good candidate for Tn5 mutagenesis. To facilitate the continuation of this study, alternative Tn5 delivery vectors such as "suicide" plasmids pSUP2021, pGS9, and pLG221, all of which carry Tn5 with genetic backgrounds different to that of pUW964, were also

employed. However, with *A. salmonicida* 80628, repeated conjugation experiments under different mating conditions using these vectors were disappointing, and not a single kanamycin-resistant colony was observed on selection plates, indicating that none of these plasmids was transferred into *A. salmonicida* 80628. The transposon carrier pSUP2021 used here either incorporates or can be mobilized by the broad-host range IncP group transfer (*tra*) genes. The *tra* genes for the mobilizable vector pSUP2021 are integrated within the chromosome of the *E. coli* donor strain (Simon *et al.*, 1983); delivery vectors pGS9 and pLG221 belong to incompatibility groups IncN and IncI_a respectively (Selvaraj & Iyer, 1983; Chatfield *et al.*, 1982). *A. salmonicida* plasmids were reported to belong to incompatibility groups IncA and IncU (Aoki *et al.*, 1986; Hedges *et al.*, 1985), thus differences in incompatibility groups between *A. salmonicida* plasmids and plasmid vectors pSUP2021, pGS9 and pLG221 could account for the unsuccessful transfer of these plasmid from *E. coli* into *A. salmonicida* 80628. "Suicide" plasmid pUW964, which incorporates the *colE1* replicon, was transmissible to *A. salmonicida*, and the vector was maintained in the recipient strain at high frequencies. Analysis of expression of both transposons (Tn5 & Tn7) carried by this plasmid, was shown in most transconjugants obtained, indicating a possible maintenance of the plasmid carrier in *A. salmonicida* 80628. These studies also indicate that all Tn5 and Tn7 resistance markers were expressed in transconjugants but in different proportions, resistance to kanamycin and streptomycin being expressed more frequently than resistance to trimethoprim and spectinomycin. Moreover, analysis of the plasmid content of transconjugants showed difference in their profiles on agarose gels when compared to the parental strain 80628, with the appearance of a 15.4 kb band, which, in respect of its molecular weight, corresponded to insertions of both Tn5 and Tn7 into the 4.4 kb plasmid of *A. salmonicida* 80628 resulting in appearance of this plasmid band of higher molecular weight. Such results indicate preferential insertion of Tn5 and Tn7 into plasmids rather than into chromosomal DNA of *A. salmonicida*.

Reversion of transconjugants to their wild type phenotype occurred at high frequency; approximately 20 % of mutants studied lost their ability to grow on kanamycin-containing BHI plates and this indicates possible rearrangement mechanisms of DNA after insertion, probably by excision of Tn5. The excision of transposon Tn5 has been reported to occur at high frequency in *Pseudomonas aeruginosa* (Goldberg *et al.*, 1990), excision of the transposon is apparently due to the presence of long inverted repeat sequences.

6.2- Expression of caseinase, gelatinase and haemolysins in *A. salmonicida* Tn5 mutants

Initially, transconjugants were selected for loss of caseinase expression due to inactivation of the caseinase gene by Tn5 insertion. Evidence for the loss of caseinase activity was first obtained on selection plates containing casein. However, these caseinase-negative mutants were unstable and activity was regained after storage of several of these mutants in 25 % BHI-glycerol solution at - 20 °C. Reversion of caseinase activity was also apparent in liquid medium, in which the caseinase activities of these mutants increased over time; less activity was released during exponential and early stationary phase when compared to their parental strain. This was probably due to alteration of caseinase translocation across the outer membranes of mutants during the early stages of their growth. Similar weak protease-producers of *A. hydrophila* obtained by Tn5 mutagenesis were reported by Leung & Stevenson (1988), probably a result of a more general export deficiency.

Haemolytic activities of weak caseinase-export mutants on horse and salmon blood were compared to those produced by their parental strain. Negligible amounts of T-lysin and H-lysin activities were observed in culture supernatants of mutants after 24 h, but these activities increased rapidly, reaching levels of activity similar to *A. salmonicida* 80628. A clear correlation between caseinase and haemolytic activities was observed among these mutants, supporting the results of Titball & Munn (1983, 1985).

Gelatinase produced by *A. salmonicida* has been considered a virulence factor (Smith *et al.*, 1980); all mutants tested here by Gelatin-PAGE analysis, produced

gelatinase. Gelatinase activities of mutants which were weak caseinase producers were not different from the activity produced by the parental strain 80628 .

Analysis of protein profiles of culture supernatants of mutants on SDS-PAGE revealed interesting differences. A major band corresponding to approximately 70 kD was observed in profiles of all mutants including weak caseinase producers, demonstrating that caseinase activity of these mutants remained in culture supernatants. Interestingly, some mutants showed another dense band of approximately 50 kD, corresponding to the A-protein, in their culture supernatants. This was not observed in the parental strain 80628.

The Tn5 insertion did not cause major changes in the mutants, which showed similar growth characteristics to their parental strain, and no virulence factors such as caseinase and haemolysins were totally abolished by these mutations. Insertion and expression of Tn5 and Tn7 was probably the main cause of transport alterations of caseinase observed in mutants which were weak caseinase producers. Transport alterations were reported in other gram-negative bacteria such as *E. coli* (Cohen *et al.*, 1988) in which multiple antibiotic-resistant mutants expressed less OmpF porins; similar studies showed that low level-resistance to antibiotics in *A. salmonicida* was associated with changes and alterations in outer membrane proteins, probably porins (Wood *et al.*, 1986; Griffith *et al.*, 1989; Barnes *et al.*, 1990). Such condition could have effects on the outer membrane permeability to entry and exit of various compounds, including nutrients, enzymes, metabolites, and antibiotics.

6.3- Transposon mutagenesis of *A. salmonicida* 4700 strain

Following unsuccessful attempts to generate mutants of *A. salmonicida* strain 80628 deficient in caseinase production by using different Tn5 shuttle vectors, *A. salmonicida* 4700 strain was then employed. This strain, isolated from an outbreak of furunculosis in Scotland, differed from *A. salmonicida* 80628 strain in its susceptibility to antibiotics, and it was used as a transposon recipient. *E. coli* BRD 327 (SM 10 λ pir) carrying "suicide" plasmid pRT733 was the TnphoA donor strain. This plasmid is a

derivative of pJM103.1 in which the *colE1* origin of replication (*ori*) has been replaced by that of plasmid RK6, and can not replicate in the absence of λ *pir*, but can be mobilized from *E. coli* SM10 λ *pir* into recipients during matings (Taylor *et al.*, 1989). This shuttle plasmid was successfully used to generate transposon mutants of *Salmonella typhimurium* by Miller *et al.* (1989).

Transposon TnphoA is a derivative of Tn5 (see section 2.2) which contains *phoA*, the *E. coli* gene for alkaline phosphatase. Random insertions of TnphoA into chromosomal DNA yield in-frame fusions between a target gene and *phoA* thus encoding a hybrid protein which has a carboxyl-terminal fragment of *phoA*, fused to an amino-terminal portion of the target protein product. These hybrid proteins display alkaline phosphatase activity only if the target gene encodes a protein expressed at the cell surface (a secreted, transmembrane, or outer membrane protein) and so provides the requisite signals for transport of the carboxyl-terminal *phoA* fragment into the periplasmic space (Manoil & Beckwith, 1985).

A. salmonicida has been reported to express virulence determinants at the cell surface (Trust *et al.*, 1983; Ishiguro *et al.*, 1981; Munn *et al.*, 1982; Sheeran & Smith, 1981; Buckley *et al.*, 1982; Titball & Munn, 1982; Fuller *et al.*, 1977) and TnphoA-generated mutants could be selected for insertions of TnphoA into genes encoding some of these virulence determinants, such as the A-layer, LPS and outer membrane proteins, in addition to loss of caseinase expression.

Mutagenesis using TnphoA has been extensively used to study virulence of *Salmonella* (Finlay *et al.*, 1988; Miller *et al.*, 1989), *Vibrio cholerae* (Taylor *et al.*, 1987; Goldberg *et al.*, 1990), *E. coli* (Donnenberg *et al.*, 1990), and also in *Erwinia amylovora* (Coleman *et al.*, 1991). In this study the shuttle plasmid vector pRT733 was used successfully to generate mutants of *A. salmonicida*. The frequencies of appearance of kanamycin-resistant colonies and spontaneous mutants to kanamycin of *A. salmonicida* strain 4700 were within the expected range for frequencies of Tn5, but different from those observed with *A. salmonicida* 80628. Selection of TnphoA transconjugants on selection plates was focused on kanamycin-resistant colonies showing a dark uniform non-diffusible

blue color. Colonies of mutants grew slowly on selection plates (up to 7 days), and their colonies were smaller than their parental strain. The use of TnphoA was advantageous for selection of mutants. However kanamycin-resistant colonies which did not produce a blue color were also observed on selection plates in low number indicating that insertions of TnphoA can generate kanamycin-resistant mutants without expressing the phoA gene. The appearance of the blue color in colonies of mutants was essentially due to phoA expression, but incubation of *A. salmonicida* 4700 on BHI agar plates containing X-P did show production of a pale blue color concentrated in the centre of colonies only. This indicates endogenous alkaline phosphatase expression in *A. salmonicida*. To avoid confusion between spontaneous mutants to kanamycin and TnphoA transconjugants, only kanamycin-resistant colonies showing a blue color were picked for further studies.

Selected mutants showed stability in kanamycin-resistance over the period of this study. Resistance to higher concentrations of kanamycin ($550 \mu\text{g ml}^{-1}$) indicated a positive insertion and expression of TnphoA into genomic DNA of the mutants.

The use of TnphoA in generating mutants was also advantageous in selection of transconjugants in liquid medium. Phenotypes of mutants in liquid medium supplemented with the chromogenic substrate for alkaline phosphatase (X-P) were different from their wild type. It is believed that alkaline phosphatase activities of mutants were higher than in *A. salmonicida* 4700; however, attempts to quantify and compare these activities failed to detect any significant differences. The expressed dark blue colour by mutants was not diffusible in liquid medium and was associated with bacterial cells, indicating cell association of alkaline phosphatase activities in these mutants. The characteristics of the activities of these mutants are similar to the alkaline phosphatase activity of TnphoA insertions reported by Manoil & Beckwith (1985).

Differences between TnphoA colonies were observed on BHI agar plates in that mutants Pa3, Pa7, Pa10, and Pa18 produced colonies with rough surfaces, but other TnphoA mutants produced colonies similar to the parental strain. Such colony characteristics were reported earlier by Duff (1937), and by Udey & Fryer (1978) who

demonstrated that aggregating, or "R" (rough), strains were more virulent than the non-aggregating "S" (smooth) strains, characteristics which were presumably due to the presence or absence of the A-layer. In this study, results indicated that appearance of rough and smooth colonies of mutants did not coincide with aggregation and non-aggregation of cells, since all mutants tested showed some autoagglutination when suspended in saline. Thus, cell surface characteristics of mutants Pa3, Pa7, Pa10, and Pa18 could be due to the presence of A-layer and to LPS exposure at the cell-surface.

No major cultural differences, apart from surface characteristics, were observed among mutants, in that growth of mutants in liquid and solid medium, during shaken and static cultures, and production of diffusible brown pigment, were similar to that of their parental strain. This indicates that mutants retained the growth ability of their parental strain without loss of expression of vital enzymes, which could impair or restrict their growth. The medium used was BHI, which is a rich medium containing nutrients and elements allowing growth of many microorganisms. Consequently, the use of such a medium could mask phenotypes that were altered following insertions of TnphoA. It would be interesting to study and compare the growth of mutants on different media with defined chemical and nutritional composition. Such studies were not included in this project because of the restricted time available.

6.4 - Evidence of TnphoA insertions into *A. salmonicida* chromosomal DNA

In this study mutants were selected for three main characteristics : resistance to kanamycin, loss of expression of caseinase and expression of alkaline phosphatase activity due to TnphoA insertions.

The selection was not done randomly, since the prime target of this study was to generate mutants deficient in caseinase expression. Thus, a number of tests were carried out to determine whether isolates were TnphoA insertion mutants or TnphoA-pRT733 - maintained mutants. Plasmid profiles of TnphoA mutants showed no clear evidence for transposon insertion suggesting a different situation from that observed in *A. salmonicida* 80628

Tn5 mutants. Also, since expression of TnphoA occurs only when the transposon is inserted into a gene target which encodes for exported protein, the production of the characteristic blue color of colonies of TnphoA mutants indicated a positive insertion and expression of the transposon into genomic DNA of *A. salmonicida*. This blue colour is essentially due to the activity of alkaline phosphatase on the chromogenic substrate X-P, however endogenous alkaline phosphatase of *A. salmonicida* 4700 was observed on solid medium supplemented with the substrate, but was not observed in liquid medium.

Detection of TnphoA insertions was carried out using the method of Southern (1975). During DNA extraction many problems were encountered, mainly problems of DNA breakage, digestion with contaminating nucleases, and resistance to restriction enzyme digestion. Several methods of extraction which were employed did not give satisfactory results, however, the "miniprep" extractions (see section 3.16.3) were satisfactory. In such situations, plasmid DNA could be mixed with chromosomal DNA resulting in confusing results in which extracted DNA of TnphoA mutants was only partially digested with *EcoR* I. This was observed on several occasions suggesting the presence of contaminating endogenous proteases of *A. salmonicida*. The aim of using *EcoR* I to digest DNA is because the structural gene of Tn5 does not contain restriction sites for this enzyme (Jorgensen *et al.*, 1979); thus, DNA fragments containing parts of the transposon could be avoided allowing detection of the entire gene. The 4.8 kb *Xho* I fragment of plasmid pGLW50 contained the structural gene of Tn5 and was used as a probe for TnphoA inserts. Single bands of hybridization with high molecular weight were observed with all mutants tested except the parental strain, indicating a positive insertion of TnphoA. However, estimation of molecular weights of these bands was not possible and this was essentially due to partial digestion of DNA as indicated above.

Further evidence of TnphoA insertion was obtained from the ability of mutants to grow in high concentrations of kanamycin. Greater than 10-fold resistance to the transposon marker (compared with the wild type) was observed in mutants tested, and this clearly indicated insertion and expression of TnphoA in *A. salmonicida*.

6.5 - Alteration of caseinase expression in TnphoA mutants

Excretion of proteases by *Aeromonas* species has been previously reported by Dahle (1969, 1971), and these enzymes were believed to be of major importance in the pathogenesis of the bacterium. Later studies on *A. salmonicida* proteases indicated that the bacterium produced two distinct proteases (see section 1.3.2.2), and the 70 kD serine protease was considered to be a major virulence factor of the organism. The exact role of this protease in the pathogenesis of furunculosis remains obscure, and speculation about its role was the subject of many reports. For example Sakai (1985) reported that protease was the most important virulence factor in furunculosis from his work using protease-deficient mutants. But, Tajima *et al.* (1987) and Hackett *et al.* (1984) found that protease-deficient mutants could cause furunculosis and that protease was not essential for causing the disease. These controversies could arise from the different methods and strains of *A. salmonicida* used by different workers. So far, one caseinolytic activity secreted by the organism has been reported; purification of the activity involved ion-exchange chromatography with assays for casein digestion. The presence in culture supernatants of caseinase activities other than that caused by the 70 kD serine protease could be obscured by the latter enzyme; the use of transposons to create mutants lacking expression of the protease is a useful means for detecting other caseinolytic activities produced by the organism. In this study, protease-negative mutants were successfully generated as results indicated that no 70 kD caseinase was expressed in culture supernatants of the mutants selected; however, a small amount of caseinase activity was observed in culture supernatants of mutants during both static and shaken culture in BHI broth. This activity was not observed on solid agar plates, suggesting that the expression of activity is governed by culture conditions and probably the culture medium used.

The gelatinase activity of mutants was not altered, suggesting that *A. salmonicida* produced at least two distinct proteases, supporting the results of Sheeran & Smith (1981) and Drinan *et al.* (1989). The persistence of caseinase activities in culture supernatants of protease-deficient mutants of *A. hydrophila* has been reported by Leung & Stevenson

(1988) who also reported that detection of such activities was achieved using a more sensitive indicator medium, thus, caseinase activities of mutants deficient in protease transport could be difficult to detect. Price *et al.* (1989) reported that *A. salmonicida* probably produced more than two proteases. Most studies on proteases secreted by *A. salmonicida* have concentrated on the 70 kD serine protease, and gelatinase had received very little attention. Characterization and further investigation of the gelatinase could be very interesting, and could throw light on the pathological significance of the enzyme. The lack of precise roles for these proteases in the pathogenesis of the disease could account for discrepancies between the reports of many workers. Drinan *et al.* (1989) reported that possession of the 70 kD protease is essential for *A. salmonicida* virulence or toxicity of its ECP, but this requirement could perhaps be met by the gelatinase produced by the organism, as these authors reported that naturally-occurring protease-negative mutants of *A. salmonicida* produced negligible caseinase activities, but retained their gelatinase activity; also, mutants remained virulent for fish but with increased LD₅₀ values. It is possible that gelatinase was involved in the activation of other virulence factors such as GCAT, haemolysins and leukotoxins which caused the death of fish. The possibility that, *in vivo*, *A. salmonicida* secreted other virulence factors such as caseinases which were repressed *in vitro* must also be considered.

In the present study, selected protease-negative mutants did not express the 70 kD serine protease, and this was demonstrated by the absence of caseinase activity on BHISMA plates, and the absence of the 70 kD protein band on SDS-PAGE and immunoblots but a small amount of residual activity was observed in culture supernatants of mutants. It is unlikely that protease-negative mutants expressed the 70 kD protease as a non-mature enzyme which was activated by the gelatinase, since immunoblotting of culture supernatants did not show any band corresponding of the non-mature enzyme. Thus, in the light of these results, ^{there is the possibility} that a second caseinase activity was detected in culture supernatants of mutants lacking expression of the 70 kD protease. The activity found was less than that by the 70 kD protease suggesting that *A. salmonicida* produced low amounts

of this second caseinase under different growth conditions; to date no other studies have reported such an activity in *A. salmonicida* culture supernatants; this could be due to the variability of strains studied by workers and the limited studies using caseinase-negative mutants.

6.6 - Analysis of other virulence factors of *A. salmonicida* TnphoA mutants

Virulence factors produced by *A. salmonicida* have been the subject of many studies, yet little is known about their roles in the pathogenesis of the disease. Such studies have involved purification of cell components and extracellular products secreted by the organism; they have mainly been considered as individual toxins, but some studies have included the interactions between these factors (Hastings & Ellis, 1985; Titball & Munn, 1985; Ellis *et al.*, 1989; Lee & Ellis, 1990; Rojso *et al.*, 1993). Here, selected mutants were characterized for several virulence factors (protease, T-lysin, H-lysin, GCAT and A-layer); three important classes of mutants were observed, the first consisting of mutants which were kanamycin-resistant which did not express the 70 kD protease, the second class included kanamycin-resistant and protease-negative mutants which secreted A-protein into culture medium, and finally, the third class represented kanamycin-resistant and protease-positive mutants which secreted the A-protein. Initially, analysis of the parental strain characteristics revealed that the strain was a protease producer and did not release the precursor of the A-layer into culture supernatant. Analysis of mutants exporting the A-protein into culture supernatants revealed that the A-protein was exported in remarkable amounts as shown by SDS-PAGE analysis. The same characteristic was observed in some Tn5 mutants of strain 80628. Among TnphoA mutants studied, four (Pa3, Pa7, Pa10, and Pa18) shared this characteristic, although they belonged to different classes of mutants. Analysis of the A-layer on congo-red and Coomassie-Blue agar plates indicated the presence of the layer on the surface of cells. Moreover, observation of thin sections of cells of these mutants revealed that the A-layer was altered rather than totally lost in mutant Pa3. Several workers reported that the A-layer represents a well-organised external protein layer,

but little information is available on the mechanisms of assembly of the protein on the cell surface. The information available to date indicates that LPS acts as a framework in which the assembly occurs but the nature and characteristics of the LPS framework is unknown. Also, metabolic pathways and enzymes involved in the process have not yet been elucidated. Studies on the assembly of the A-layer, using Tn5 mutagenesis (Belland & Trust, 1985) demonstrated that alteration of LPS expression resulted in export of the A-protein in large amounts into the culture medium. Thus, results obtained in this study supported those of Belland and Trust (1985). Mutants Pa3, Pa7, Pa10, and Pa18 were all shown to be resistant to phages specific for *A. salmonicida* and which bind LPS. In addition, SDS-PAGE analysis of extracted LPS indicated loss of high molecular weight smooth LPS which presumably represent receptors for the phages used in this study. Analysis of LPS synthesis in *A. salmonicida* has indicated that the enzyme CTP: CMP-3-deoxy-manno-octulosonate cytidylyltransferase (CMP-KDO synthase) was involved in the process. The enzyme was inhibited by the antibacterial agent compound IV (α -C-[1,5-anhydro-7-amino-2,7-dideoxy-D-manno-heptopyranosyl] carboxylate in amide linkage to the carboxyl terminus of L-alanylalanine) resulting in accumulation of new metabolites, and cessation of growth (Goldman *et al.*, 1992). The use of specific bacteriophages in the isolation of *A. salmonicida* strains lacking LPS has been reported by Popoff (1971) and Rodgers *et al.* (1981). This method proved to be a useful criterion in epidemiological studies, and the above authors demonstrated that LPS were effectively receptors of bacteriophages. It was assumed that the O-polysaccharide chains of LPS would act as the bacteriophage receptors, as only these chains penetrate the A-layer, and are exposed on the external surface of the cell; the core oligosaccharide and lipid A moieties are therefore inaccessible for bacteriophage adsorption as they are masked by the A-layer. However the isolation of a bacteriophage which was specific for the lipid A moiety was reported by Ishiguro *et al.* (1983). This indicates a possible exposure of diverse parts of the LPS molecule on the cell surface. It is very likely that LPS receptors for bacteriophages reported by these authors corresponded to those which were not expressed in some of the mutants

obtained in this study (mutants Pa3, Pa7, Pa10, and Pa18). The loss of expression of LPS was associated with rough colony surface characteristics of these mutants.

In the light of these results, TnphoA insertions affected organization of the A-protein on the surface of cells but did not cause loss of the A-layer. Analysis of mutants Pa1 and Pa2 indicated probable transport alterations due to the mutations. Protein profiles of the outer membrane of these mutants showed the presence of large amounts of a protein band corresponding to the A-protein; this characteristic was not observed in other classes of mutants studied and these alterations were probably caused by loss of expression of certain enzymes required for translocation of the A-protein across the outer membranes. However, protein profiles of outer membranes of these mutants were similar to those of their parental strain, indicating that proteins and enzymes involved in transport of the A-protein were probably not associated with the outer membrane. Moreover, the LPS content of these two mutants was similar to strain 4700 but different from mutants Pa3, Pa7, Pa10, and Pa18, which lacked expression of LPS. Thus, LPS was not involved in translocation of the A-protein across the outer membrane. Taking these data into account, the probable regulatory mechanisms involving expression of protease, LPS, and transport and assembly of the A-protein were altered as a result of TnphoA insertion, resulting in the different phenotypes observed in this study. This is the first report in which alteration of protease expression and disruption of the integrity of the A-layer together have been observed.

Evidence of disruption of the integrity of the A-layer was observed in mutant Pa3 only; mutants Pa7, Pa10, and Pa18, which secreted the A-protein into liquid medium, did not show differences in surface characteristics when compared to their wild type. These observations suggested that the A-layer was a multilayer and not a monolayer structure, supporting the results reported by Thornton *et al.* (1991); these authors reported that mutants with a disrupted cell surface may be at an advantage since they may expose critical cell-surface protective antigens normally tightly shielded by the highly organised A-layer. The mechanisms of synthesis, transport, and assembly of the A-layer are still unknown. Belland & Trust (1985) reported that at least one gene product was necessary for transport

of A-protein across the outer membrane of the bacterium. In a recent report, Chu *et al.* (1993) identified and characterized a gene which influences expression of the A-layer gene (*vapA*). The new gene, termed *abcA* gene, encoded for a 34 kD protein which is believed to belong to the ABC transport family of proteins, or so-called traffic ATPases, found in most bacteria. The protein was bifunctional, with regulatory and transport activities as well as an ATPase activity. The gene appears to be conserved in both typical and atypical strains of *A. salmonicida* and was not present in other tetragonal S-layer producing *Aeromonas* strains (*A. hydrophila*, and *A. veronii*). The gene mapped immediately downstream of the surface array protein structural gene, and appeared to be followed by a gene encoding for an outer membrane protein. However, the above authors indicated that *vapA* and *abcA* genes do not appear to be arranged in a transcriptional unit; they are separated by a sizeable DNA sequence and the two genes have significantly different codon usage. The *abcA* gene expressed itself from its own promoter, and the presence or absence of the gene clearly influenced production of the A-protein of *A. salmonicida* in *E. coli*. The same authors reported that the appropriate location of the *abcA* gene, and its product may influence production and transport of either the A-protein, and other surface or extracellular components such as a surface carbohydrate. Such observations indicate that *A. salmonicida* possesses a rather general transport mechanism for its virulence factors, and alterations of these mechanisms result in accumulation of exported proteins. This suggestion could probably explain characteristics of weak caseinase producers of *A. salmonicida* 80628 obtained by Tn5 insertions in this study. So far, three genes involved in expression and transport of the A-protein have been identified (Belland & Trust, 1987; Chu & Trust, 1993) and it is likely that other gene products are involved in the assembly of the A-protein on the bacterial cell surface.

Studies on S-layer of *A. hydrophila* have also indicated that LPS is involved in maintenance of this layer in the same fashion as for the A-layer of *A. salmonicida*; loss of the LPS resulted in release of the S-layer and its precursor, and its inability to anchor on the cell surface. Moreover, Dooley & Trust (1988) reported that a complete side-chain profile

of LPS produced by *A. hydrophila* was not an absolute requirement for anchoring an intact S-layer to cell surface, suggesting that other components in addition of LPS are necessary for assembly of the protein layer. Also, mesophilic aeromonads (*A. hydrophila*, *A. sobria*) displayed different characteristics of their surface protein layer in regard to congo red binding and hydrophobicity as in the case of *A. salmonicida* A-layer (Kokka *et al.*, 1991). Analysis of amino acid sequences of A-protein produced by *A. salmonicida* and *A. hydrophila* showed no homology over the first 30 residues (Dooley *et al.*, 1988), thus, *A. salmonicida* seems to produce a unique type of S-layer specific to this species only.

Analysis of expression of H-lysin by TnphoA mutants revealed the relationship between the 70 kD serine protease and the H-lysin which was reported by Titball and Munn (1981), and supported by Hackett *et al.* (1984) and Hastings & Ellis (1985). Results obtained in this study indicate that H-lysin was expressed in an inactive form which was activated by proteolytic cleavage; it seems that the activity of H-lysin was expressed by the wild type during both static and shaken conditions, and there was no apparent difference in its expression under these conditions, thus, these results are in contrast to the findings of Titball (1983) who stated that production of H-lysin was favoured by growth conditions of *A. salmonicida*. TnphoA mutants lacking expression of the 70 kD protease showed no lysis of washed horse erythrocytes on agar plates, but they expressed less activity when grown in liquid medium with shaking. In their studies, Titball & Munn (1985) indicated that caseinase-negative mutants of *A. salmonicida*, obtained by chemical mutagenesis using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, produced indistinct haemolysis on agar plates containing erythrocytes when compared to their parental strain, but no activity of the H-lysin was observed in culture supernatants of these same mutants. It is clear that H-lysin activities of TnphoA mutants obtained in this study was not completely impaired as a result of loss of the 70 kD caseinase, and it is probable that activation of the pro-H-lysin of these mutants was due to other protease(s) expressed by caseinase-negative mutants; this also confirmed the presence of more than one caseinase activity in culture supernatants of *A. salmonicida*. The reduced H-lysin activities produced by these mutants could be related to

the amount of caseinase present in their culture supernatants; the appearance of this activity during shaken but not during static conditions is not understood.

Further investigations showed that all mutants obtained in this study expressed activities against washed salmon erythrocytes and these activities probably corresponded to the T-lysin reported by Titball & Munn (1981). Analysis of these activities showed that mutants released the lytic activity into their culture supernatants; also, these activities were observed during both shaken and static cultures. On salmon blood plates, mutants produced clear zones of haemolysis when compared to their parental strain but differences were observed on these plates for mutants expressing, and mutants lacking, the 70 kD protease. However, titration of these activities revealed considerably reduced amounts produced by caseinase-negative mutants when compared to the wild type and protease-positive, kanamycin-resistant mutants. It was clear that T-lysin was expressed with reduced activity by caseinase-negative mutants; this ^{might suggest} that the 70kD caseinase was important in enhancing the activity of T-lysin. These results support those reported by Titball & Munn (1983, 1985) which indicated that complete lysis of trout erythrocytes by T-lysin required the 70 kD caseinase. It is also possible that the second caseinase activity and/or gelatinase observed in culture supernatants of the 70 kD caseinase-negative mutants were involved in lysis of salmon erythrocytes. The presence of reduced activities of the T-lysin in culture supernatants of mutants indicates that alteration in transport of the toxin may have occurred; similar observations were found for H-lysin of the same mutants. Titball & Munn (1983) indicated that T₁ activity produced incomplete lysis of trout erythrocytes rather than reduced haemolytic activity of the T-lysin. They also reported that caseinase-negative strains of *A. salmonicida* produced lysis of trout erythrocytes in agar plates; the exact role and mechanisms of T₁ activity reported by Titball & Munn (1983) need to be elucidated; results obtained here indicated that a decrease of activity due to lack of activation of T-lysin rather than an incomplete lysis of salmon erythrocytes. It may be that trout erythrocytes are less susceptible to T-lysin than the salmon erythrocytes. Rockey *et al.* (1988) investigated the *in vitro* relationship between caseinase and T-lysin and found that T-lysin

was responsible for outer-membrane disruption, which was enough to cause lysis, and that caseinase acted on the nuclear membrane. They also suggested that the two activities were independent and that RBC from different species were lysed by purified T-lysin only, indicating that the component responsible for incomplete lysis of trout erythrocytes reported by Titball & Munn (1983) is similar to T-lysin is still not clear. In their recent report Hirono and Aoki (1993) reported the nature and number of haemolysins synthesized by *A. salmonicida*, they indicated that the organism possessed at least three haemolysin genes, of which ASH1 gene was unique to *A. salmonicida* and the ASH3 gene encoded a haemolysin which belongs to the aerolysin family. These authors are the first to report that an aerolysin-like toxin could be produced by the organism, and the three cloned haemolysins differed from the H-lysin because of differences in their molecular weights, and were different from the T-lysin in respect of their specific haemolytic activities. This is in contrast with the results found in this study. The low levels of H-lysin, observed in liquid medium, and the T-lysin activities could perhaps be due to the haemolysins reported by Hirono and Aoki (1993).

Vibrio species have been reported to release an unusual lipolytic enzyme (MacIntyre *et al.*, 1979), which shares several properties with plasma lecithin:cholesterol acyltransferase (LCAT). Studies of mutants of *A. salmonicida* expressing the cloned GCAT gene of *A. hydrophila* (Hilton *et al.*, 1990) indicated that the activity of the enzyme was similar to that of *A. salmonicida* GCAT. These authors isolated and sequenced the GCAT gene of *A. hydrophila*, and indicated that its predicted molecular weight of 31.3 kD (Thornton *et al.*, 1988) was higher than the molecular weight of purified *A. salmonicida* GCAT (23.6 kD; Buckley, 1982). Previous work indicated that *A. hydrophila* GCAT involved more than one enzyme and these were isolated together as a high-molecular-weight complex of over 500 kD (MacIntyre & Buckley, 1978). A recent analysis of *A. salmonicida* GCAT showed that the enzyme had a molecular weight of 25 kD and occurred in ECP as a high-molecular-weight complex similar to the GCAT produced by *A. hydrophila* (Lee & Ellis, 1990). The *A. salmonicida* GCAT was associated with LPS

which stabilized and enhanced its activity; the nature of this relationship has still to be elucidated, but no activation of *A. salmonicida* GCAT by proteolytic cleavage has been reported.

Lee and Ellis (1990) showed that GCAT associated with LPS produced higher haemolytic activity than GCAT alone and they also suggested that the GCAT-LPS complex was a major lethal exotoxin and cytotoxin of *A. salmonicida*. The exact nature of the GCAT-LPS association is still unclear, but these authors postulated that LPS might enhance the haemolytic activity of GCAT by aiding the enzyme to penetrate the cell membrane. They also showed that LPS protected GCAT from proteolytic attack *in vivo* and thus could enhance the toxic effect of the complex. In a recent study, Lee and Ellis (1991) indicated that the leucocytolysin described by Fuller *et al.* (1977), the cytotoxin described by Cipriano (1982), and T₁ activity described by Titball and Munn (1985) may be similar to the GCAT-LPS complex. It is also very likely that the salmolysin described by Nomura *et al.* (1988), which was produced by *A. salmonicida* as a high molecular weight glycoprotein, corresponds to the GCAT-LPS complex.

The mechanisms of haemolysis of GCAT or GCAT-LPS remains to be elucidated; Lee and Ellis (1990) indicated that GCAT-LPS was selectively lytic for fish and not for rabbit erythrocytes, probably due to the phospholipid composition of the erythrocytes membranes, as fish tissues are richer in polyunsaturated fatty acids than those of mammals (Lee *et al.*, 1989). In a very recent report, Rosjo *et al.* (1993) indicated that lysis of salmonid red blood cells resulted in accumulation of lysophospholipids in the membrane following enzymatic action of the GCAT-LPS complex. However, this activity was not observed with human erythrocytes, probably due to differences in membrane composition and/or rapid degradation of lysophospholipids.

Most studies on the phospholipase activity expressed by *A. salmonicida* indicated the presence of phospholipase A₂-like activity, but Campbell *et al.* (1990) reported that the phospholipase activity detected in culture filtrates was essentially a phospholipase C activity distinct from either GCAT or haemolysin activity. These authors used a simple assay with

the chromogenic substrate trinitrophenyl-phosphatidylcholine compared to the assays used by others to study GCAT activity. The assay of Campbell *et al.* (1990) could not differentiate between phospholipase A₂ and phospholipase C activities since the assay was based on the release of phosphate with parallel release of the trinitrophenyl chromagen. Such releases could easily due to GCAT and endogenous alkaline phosphatase.

Analysis of phospholipase C produced by different species of bacteria, such as *Pseudomonas fluorescens*, *Clostridium perfringens*, *Bacillus cereus*, *Bacillus thuringiensis* and *Staphylococcus aureus* showed that the activity of the enzyme required divalent cations such as Ca⁺⁺ and Mg⁺⁺ (Waite, 1987), a characteristic not observed with phospholipase A₂. In this study, the lecithinase observed on lecithin-agar plates was a phospholipase A₂, since stimulation and inhibition tests using Ca⁺⁺ and EDTA respectively, did not alter the activity, whereas phospholipase C of *C. perfringens* was stimulated by Ca⁺⁺ and completely inhibited by EDTA. The phospholipase activity observed in culture filtrates of mutants which did not express the 70 kD protease was less than that produced by the parental strain 4700, indicating that these mutants were unable to produce high amounts of GCAT observed in strain 4700. This suggested that the 70 kD protease was important in producing high amounts of the enzyme. It is also possible that activation of GCAT by limited proteolysis was necessary; this mechanism was suggested by Hilton *et al.* (1990) who cloned the GCAT gene of *A. hydrophila* into an *A. salmonicida* strain and showed that proteolysis of the expressed enzyme was necessary for its activation. The reduction of GCAT activity in mutants studied here coincided with the absence of the 70 kD protease and the detection of the second caseinase activity in culture filtrates (discussed earlier in this section). It is possible that a second caseinase activity was responsible for activating the limited amount of GCAT activity which was detected. It would be of interest to add purified protease to culture filtrates of these mutants, to determine whether proteases effectively are involved in the activation of GCAT. Lee and Ellis (1989) indicated that purified protease was lethal in large doses, and combinations of protease and GCAT-LPS complex showed an additive relationship in lethality. The nature of this relationship was not

investigated, but results obtained here support those of Hilton *et al.* (1990) and Lee and Ellis (1989). Moreover, reduced haemolytic activity against salmon erythrocytes observed in this study with certain mutants was probably due to limited activity of GCAT; this tends to support the hypothesis that the T1 activity is similar to GCAT (Lee and Ellis, 1990).

The characterization of caseinase-negative mutants obtained in this study involved the analysis of several potential virulence determinants in order to determine the relationship between these factors. It is well recognized that understanding the interaction between the different virulence determinants of *A. salmonicida* and the interactions of the organism with the host is required in future work towards an effective furunculosis vaccine. Mutants characterized in this study exhibited different phenotypes as a result of mutations which occurred within different genes involved in the expression and the control of the 70 kD caseinase produced by the organism. In this study, disruption of caseinase expression was accompanied by disruption of the organization of the A-protein on the cell surface which was essentially due to the lack of expression of high molecular weight LPS (Table 17). This suggests ^{For the phenotypes in mutants} a possible common regulatory system between caseinase and enzymes for LPS synthesis/assembly which needs further investigation. Also the activation of the H-lysin, and probably the T-lysin or GCAT, by the caseinase was established. The study of caseinase-negative mutants also revealed that other proteases, including the gelatinase, could be expressed in culture fluids of these mutants and, consequently these proteases could play an important role in activation of other virulence determinants. It would be interesting to study *in vivo* the reactivity of isolated virulence factors produced by these caseinase-deficient mutants activated with different commercial proteases, and this would help to determine the exact role of the 70 kD caseinase in the activation of other toxins produced by the organism. The characterization of caseinase-negative mutants suggested that these mutants could be possible candidates for vaccine studies in fish, as the lack of expression of the 70 kD caseinase in these mutants will probably restrict the growth of the organisms in fish (assuming that the the 70 kD caseinase was the only means for providing these organisms with essential amino acids). Consequently this may elicit higher levels of antigenic

Table 17: Differentiating characteristics of *A. salmonicida* TnpA mutants.

	Resistance to Km	Caseinase	H-lysin	T-lysin	Export of A-protein	Loss of high M.W. LPS	Resistance to phages	Autoagglutina- tion in saline
4700	-	+	+	++	-	-	-	++
Pa1	+	-	-	(+/-)	-	-	-	++
Pa2	+	-	-	(+/-)	-	-	-	++
Pa3	+	-	-	(+/-)	+	+	+	+
Pa7	+	-	-	(+/-)	+	+	+	+
Pa10	+	-	-	(+/-)	+	+	+	+
Pa18	+	+	+	++	+	+	+	+

stimulation in fish. In a recent report, Vaughan *et al.* (1993) reported that an aromatic-amino acid dependent mutant of *A. salmonicida* can act as live vaccine for furunculosis. They described a strain of *A. salmonicida* with a mutation which affected several key metabolic pathways without affecting its ability to produce virulence factors, including the A-layer. Although analysis of virulence determinants of this mutant was not reported, these authors found that injection of fish with high doses of the mutant resulted in induction of a high level of immunity.

In vivo studies of *A. salmonicida* are needed to provide a more complete understanding of the interaction of the organism with the host and identify roles of the different virulence determinants. Garduno *et al.* (1993), using a technique which allows the study of the organism within the host, reported that *in vivo* grown cells of *A. salmonicida* expressed a polysaccharide capsular layer. Such findings were also reported by Garrote *et al.* (1992) and the role of this polysaccharide capsule layer is not understood. Garduno *et al.* (1993) also reported that strains grown *in vivo* showed differences compared to the same strains grown *in vitro* in respect of their association with rainbow trout tissue macrophages and oxidative killing. These authors suggested that virulence factors of *A. salmonicida* could be expressed in a programmed sequential response to the host environment and consequently suggested that the polysaccharide capsular layer may be important in virulence. Moreover, *in vivo* grown *A. salmonicida* cells expressed antigens which were not observed *in vitro*, and it is believed that persistence of *A. salmonicida* strains in fish is probably due to these *in vivo* -expressed antigens rather than just to the persistence of the organism (Thornton *et al.*, 1993). This indication opens new prospects for the design of an effective furunculosis vaccine. It is also important to determine how these novel antigens can be expressed *in vitro*, such studies will surely contribute greatly to the understanding of the biology of the organism.

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Appendices

Appendix IAntibiotic stock solutions

These were prepared as shown in Table:

Agent	Solvent	Concentration of stock solution	Sterilization & Storage
Furazolidone	50 % ethanol	1 mg/ml	Filtration; Autoclave at low temperature; store at -20°C.
Kanamycin	Sterile distilled water	1 mg/ml	Filtration; store at -20°C.
Streptomycin	“ “ “	0.5 mg/ml	Filtration; store at -20°C.
Spectinomycin	“ “ “	0.5 mg/ml	“ -20°C.
Trimethoprim	50 % ethanol	0.5 mg/ml	“ -20°C.
Nalidixic acid	Sterile distilled water	1 mg/ml	“ -20°C.

Preparation of BCIP (5-bromo-4-chloro-3-indoyl phosphate) :

Dissolve 5 mg of BCIP in 0.5 ml of 100 % dimethylformamide and store at 4°C. Do not sterilize by autoclaving or filtration.

Appendix II

Tris-EDTA buffer (TE):

10 mM Tris-HCl	pH 8.0
1 mM EDTA	

Equilibrated phenol / chloroform:

Phenol was equilibrated three times with an equal volume of 0.5 M Tris-HCl pH 8.0, allowing the aqueous layer to separate and to be removed. Equilibrated phenol was mixed with an equal volume of chloroform.

Tris-Borate-EDTA buffer:

90 mM Tris-base	
90 mM Boric acid	
2.5 mM EDTA	pH 8.2

50 x Denhardt's solution:

0.1 % (w/v) bovine serum albumin (BSA) fraction V (Sigma).
 0.1 % (w/v) Ficoll.
 0.1 % (w/v) pPolyvinylpyrrolidone.
 6 x SSC.

Oligonucleotide-labelling buffer (OLB):

Four solutions were made separately :

Solution O :	Tris-HCl (pH 8.0)	1.25 M
	MgCl ₂	1 mM
Solution A :	To 1 ml of solution O were added :	
	18 μ l 2-mercaptoethanol	
	5 μ l dCTP	

5 μ l dGTP

5 μ l dTTP

(All triphosphates were at a concentration of 100 mM in 0.2 mM EDTA, 3 mM Tris-HCl pH 7.0)

Solution B : 2 M Hepes buffer adjusted to pH 6.6 with 4 M NaOH

Solution C : Random hexadeoxyribonucleotides suspended in TE buffer
at 90 OD U/ml.

To make OLB buffer, solution A, B and C were mixed in the ratio 10 : 25 : 15. The buffer was stored at -20 °C.

20 x SSC:

3 M NaCl.

0.3 M Trisodium citrate pH 7.0.

CTAB/NaCl solution (10 % CTAB in 0.7 M NaCl):

In 80 ml of dH₂O was dissolved 4.1 g of NaCl. The solution was heated at 65 °C and 10 g CTAB (Sigma) slowly added to it with constant mixing. Once dissolved, the final volume was adjusted to 100 ml.

Denaturation solution :

1.5 M NaCl

0.5 M NaOH

store at room temperature.

Prehybridization solution :

25 mM potassium phosphate buffer pH 7.4

5 x SSC

5 x Denhardt's solution

50 μ g/ml salmon sperm DNA

50 % formamide

store solution at - 20°C and shake before using.

SDS/prehybridization solution :

Add 1% SDS to prehybridization solution.

SDS/hybridization solution :

Add 10% dextran sulphate to SDS/prehybridization solution.

Hybridization solution :

Combine ingredients of prehybridization solution except H₂O. Add 50 g dextran sulphate (for 10% final concentration) and mix overnight. Bring to 500 ml with H₂O.

Neutralization solution :

3 M NaCl

0.5 M Tris-HCl

pH 5.0

Store at room temperature.

Alkaline Lysis Buffer for Minipreparations of Plasmid DNA :

Solution I

50 mM glucose

25 mM Tris-HCl (pH 8.0)

10 mM EDTA (pH 8.0)

Autoclave solution and store at 4°C.

Solution II

0.2 N NaOH (freshly diluted from a 10 N stock)

1 % (w/v) SDS.

Solution III

5 M potassium acetate

60 ml

glacial acetic acid

11.5 ml

H₂O

28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

Appendix III

Polyacrylamide gel electrophoresis (Laemmli 1970):

Stock solutions:

Acrylamide / Bis:

Acrylamide	30 g
N,N-bis-methylene acrylamide	0.8 g
Distilled water	100 ml

Lower buffer:

Tris	18.1 g
SDS	0.4 g
Distilled water	70 ml

Adjust pH to 8.8 with concentrated HCl and make up the final volume to 100 ml with distilled water.

Upper buffer:

Tris	6.06 g
SDS	0.4 g
Distilled water	70 ml

Adjust pH to 6.8 with concentrated HCl and make up the final volume to 100 ml with distilled water.

Solubilizing buffer for proteins:

Glycerol	10 ml
2-mercaptoethanol	5 ml
SDS	3 g
Bromophenol blue	0.01 g

Upper buffer (1 in 8 dilution)	100 ml
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Solubilizing buffer for LPS:

Glycerol	10 ml
Sucrose	20 g
SDS	2 g
2-mercaptoethanol	5 ml
Bromophenol blue	0.01
Upper buffer (1 in 8 dilutions)	100 ml

Running buffer:

Tris	6.06 g
Glycine	28.8 g
SDS	2 g
Adjust pH to 8.3 with concentrated HCl.	
Distilled water	2000 ml

Coomassie blue staining solution:

Coomassie blue R250 (BDH)	1.25 g
50 % (v/v) methanol	454 ml
Glacial acetic acid	46 ml

Destaining solution :

Methanol	50 ml
glacial acetic acid	75 ml
Distilled water	875 ml

Slab-gel preparation:Lower separating gel:

	<u>12.5 %</u>	<u>10 %</u>
Lower buffer	10 ml	10 ml
Distilled water	13.4 ml	16.6 ml
Acrylamide/bis	16.6 ml	13.4 ml
After degassing for 20 min the following were added:		
Ammonium persulfate	200 μ l	200 μ l
(10 % freshly prepared in distilled water)		
Temed (undiluted)	20 μ l	20 μ l
Adjust to pH 8.8 with concentrated HCl.		

Upper stacking gel (4.5 %):

Upper buffer	2.5 ml
Distilled water	6 ml
Acrylamide / bis	1.5 ml
After degassing the following were added:	
Ammonium persulfate (10 %)	30 μ l
Temed	20 μ l
Adjust to pH 6.8 with concentrated HCl.	

Fixative & destain solution for G-PAGE:

Methanol	300 ml
Acetic acid	300 ml
Distilled water	300 ml.

Appendix IV**SM buffer**

1 M Tris-HCl pH 7.5	50 ml
NaCl	5.8 g
4.7H ₂ O	2 g
2 % (w/v) gelatin	5 ml

Distilled water was added up to 1000 ml.

Transfer buffer

Tris	7.5 g
Glycine	36 g
Methanol	500 ml
Distilled water up to	2500 ml

TTS buffer

1 M Tris-HCl (pH 7.2)	2 ml
Tween 20	1 ml
Saline	97 ml.

Substrate preparation for staining immunoblots

0.03 % (w/v) DAB (3-3 diaminobenzidine tetrahydrochloride dihydrate, Aldrich Chemical) dissolved in 100 ml of distilled water containing 1 % (w/v) CoCl₂, 100 μ l of hydrogen peroxide (H₂O₂) were added to the mixture immediately before use.

